University of Alberta

IDENTIFICATION, CHARACTERIZATION, AND DIFFERENTIATION OF ISLET PROGENITOR CELL POPULATIONS DERIVED FROM THE ADULT HUMAN PANCREAS

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Doctor of Philosophy in Experimental Surgery

Department of Surgery

Edmonton, Alberta, Canada

Fall 2004

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This is dedicated to Sacha Sprague; who inspired me and put up with all the long days, and to my parents; who always encouraged but never pushed me to become who I am today.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Dr. Greg Korbutt for his support and guidance in both my research and career decisions. You have been a mentor to me for these years and have provided me with opportunities I would never otherwise have had. I would also like to thank Dr. Ray Rajotte for his supervision and for always making everyone feel at ease in the lab, even when conditions are at their most strenuous. My gratitude also goes to Dr. Jonathan Lakey, Dr. James Shapiro, Dr. Gina Rayat, and Dr. Jannette Dufour for providing valuable insight, advice, and criticism on various aspects of my research. Finally, many thanks go to Dr. Rhonda Bell and Dr. Bruce Verchere for taking the time to critically review this manuscript and be a part of my final defense committee.

I am in the deepest debt to all of the staff, technicians, and fellow students of the Surgical-Medical Research Institute, without whom I could never have completed this research. Special thanks go to Dawne Colwell for invaluable assistance with presentations, figures, and formatting. Also many thanks to Rosemarie Henley and Colleen Ruptash for administrative assistance. Support in my research has come from too many people to name, but special thanks goes to James Lyon, Jennifer O'Hara, Sharleen Imes, Tatsuya Kin, Karen Seeberger, Alana Eshpeter, Tanya Binette, and Lynette Elder. I would also like to acknowledge the financial support of the Stem Cell Network of Canada.

Finally, I would like to thank the person who has supported me the most through the last four years – Sacha Sprague. You have been a sounding board, a voice of reason, and most importantly the most amazing escape from this research when I have needed it

the most. Also my parents, who have supported me emotionally, spiritually, and financially to this point in my life, and have now become friends rather than caregivers. Finally, I would like to thank my friends, who have been around so long that they are now family, and who have all inspired me in different ways that they probably do not realize. A good part of who I am today comes from you guys.

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LIST OF ABBREVIATIONS

ABC	Avidin-Biotin Complex
AIRarg	Acute Insulin Response to Arginine
AIRg	Acute Insulin Response to Glucose
ANOVA	Analysis of Variance
AUCins	Area Under the Curve for Insulin
bFGF	Basic Fibroblast Growth Factor
bHLH	Basic Helix-Loop-Helix
BSA	Bovine Serum Albumin
CK	Cytokeratin
CMV	Cytomegalovirus
CNS	Central Nervous System
Ct	Cycle threshold
DAB	3,3 diaminobenzidinetetrahydrochloride
DBA	*Dolichos Biflorus* Agglutinin
dNTP	deoxy-nucleotide triphosphate
DTT	Dithiothreitol
EGF	Epidermal Growth Factor
ES	Embryonic Stem (cell)
EGTA	Ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate

GFP	Green Fluorescence Protein
HBSS	Hank's Balanced Salt Solution
HCAM	Homing Cell Adhesion Molecule
HNF-6	Hepatocyte Nuclear Factor-6
HSA	Human Serum Albumin
IE	Islet Equivalent
ITS	Insulin-Transferrin-Selenium
IVGTT	Intravenous Glucose Tolerance Test
MAPC	Multipotent Adult Progenitor Cell
MOI	Multiplicity of Infection
NGF	Nerve Growth Factor
Ngn3	Neurogenin-3
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDX-1	Pancreatic and Duodenal Homeobox Gene-1
SD	Standard Deviation
STZ	Streptozotocin
TGF-β	Transforming Growth Factor-β
TUNEL	TdT-mediated dUTP Nick-End Labeling
UW	University of Wisconsin

CHAPTER 1

GENERAL INTRODUCTION*

I-A) INTRODUCTION TO TYPE 1 DIABETES MELLITUS

Type 1, insulin dependent diabetes is a complex and, as yet, little understood disease that affects millions worldwide. The disorder, which is characterized by juvenile onset of severe insulin deficiency due to loss of pancreatic islet cells, is believed to be caused by a combination of both genetic and environmental factors (1, 2) and persists throughout the life of an afflicted individual. Long term complications associated with type 1 diabetes are severe and result from fluctuating glycemia levels (3). These consist of problems including hypertension (4), nephropathies (5, 6), neuropathies (7), and retinopathies (8) and often lead to early mortality in these patients (9).

The pathophysiology of type 1 diabetes has been attributed to an autoimmune disorder whereby the insulin producing β-cells of the pancreas are selectively destroyed, likely due to the perceived immunogenicity of self-antigens present on the surface of these cells (10). Treatment of the disease thus stems from the need to replace circulating insulin that have been lost as well as prevent the development of potentially lethal ketoacidosis (11). The discovery and purification of insulin in 1921 by Banting and Best has provided a treatment that has remained the clinical standard, and daily injections of exogenous insulin have allowed patients with type 1 diabetes to live long and relatively normal lives. Results from the Diabetes Control and Complications Trial (12) demonstrated that intensive insulin therapy and strict blood glucose monitoring can

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** A version of this chapter has been published: Street et al. 2004 Curr. Topics in Dev. Biol. 58:111-136.*

control glycemic fluctuations and limit diabetes related complications. However, the increased risk of severe hypoglycemia as well as problems with patient compliance to intensive injection regimens means that even the best case scenario of exogenous insulin therapy does not afford the glycemic control provided by a normally functioning pancreas.

In an attempt to better control glycemia levels, transplantation of the whole pancreas has been performed with successful outcome. These transplants can provide stable and continuous normoglycemia (13), and combined kidney-pancreas transplant can be effective in diabetic patients manifesting end-stage renal disease. However, the morbidity and mortality rates owing to the extreme invasiveness of the procedure, the need for lifelong immunosuppression, and the complicated nature of the surgery limit the usefulness of pancreas transplantation to only the most severe cases of type 1 diabetes. The advantages and disadvantages of both insulin injections and whole pancreas transplantation has led to the concept of transplantation of only the pancreatic endocrine cells within the islets of Langerhans.

I-B) Islet transplantation: Success with limitations

Another therapeutic alternative for people with type 1 diabetes is the transplantation of isolated pancreatic islets to achieve insulin independence. The separation of endocrine islets from surrounding exocrine tissue was first attempted by microdissection in 1964 (14) and was later improved on by Lacy and Kostianovsky (15) who used intraductal distention with collagenase followed by mechanical and enzymatic disruption to isolate rat islets. This general protocol of collagenase digestion is still in use for human islet isolation, with the added step of islet purification achieved by centrifugation on ficoll gradients (16). Figure 1 depicts islets within intact human pancreatic biopsies (A) and in a purified clinical islet preparation (B). The procedure of islet transplantation, although successful initially in small animal models (17, 18), proved to be difficult in humans. The main reason for this is the difficulty in obtaining islet-enriched preparations from the more fibrous human pancreas (19). In results reported by the International Islet Transplant Registry (20), over 267 islet transplants were performed between 1990 and 1998, however only 12.4% of these resulted in insulin independence for periods of 1 week or more.

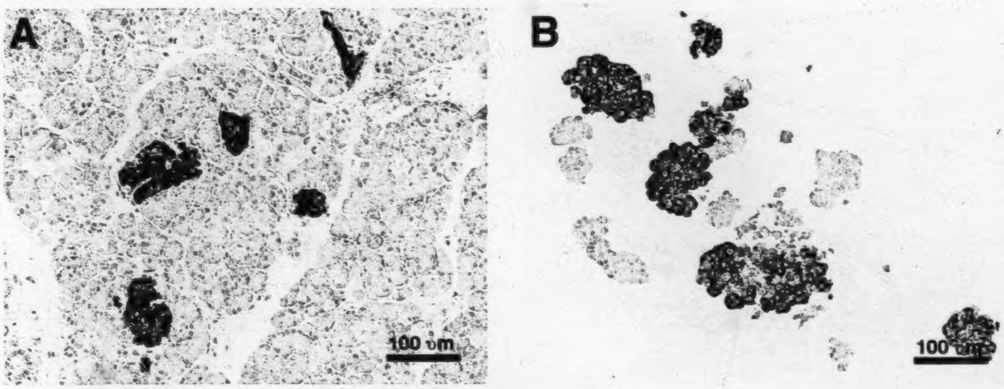

Figure 1-1: Insulin immunostaining and hematoxylin counterstain to show the presence of islets of Langerhans in human pancreatic biopsies **(A)** and purified clinical human islet grafts **(B)** (magnification: 200X).

In 2000, Shapiro and co-workers in Edmonton, Canada reported a 100% success rate in achieving insulin independence through islet transplantation in 7 long-term diabetic patients (21). Reasons for the dramatically increased success rate included a new immunosuppressive regimen excluding islet toxic glucocorticoids and the transplant of at least 10,000 islet equivalents per kilogram body weight. In recent follow up studies on the Edmonton Protocol (22, 23), a total of 12/15 (80%) of patients maintained insulin independence beyond one year post-transplant with minimal side effects. However, although clinically successful, the widespread use of islet transplantation for treatment of type 1 diabetes has been limited by several major factors. First of all, the risks associated with the lifelong immunosuppression required to prevent graft rejection may outweigh those associated with daily insulin therapy in all but the most severe cases of diabetes. Secondly, the reliance on imperfect isolation techniques and limited availability of cadaveric donor organs has resulted in an extreme shortage of transplantable islets. For this reason, there is a need to find an alternative source of islets, either as a supplement to or replacement for the small number available from cadaveric donor organs.

I-C) Alternative sources of transplantable islets

An inadequate supply of islet tissue represents a major obstacle to the widespread implementation of islet transplantation. One possibility for an alternative source of islets involves a xenogeneic supply, whereby islets from another species could be used for transplantation to humans. In this regard, porcine islets are the most attractive alternative due to physiological similarities between pigs and humans as well as the fact that porcine insulin differs from human insulin by only one amino acid (24). The extreme difficulty, however, of isolating viable adult porcine islets (25, 26) has hampered progress in this area. To circumvent this problem, methods have also been developed for the isolation and *in vitro* maintenance of neonatal porcine pancreatic cells (27) and evidence suggests that this immature tissue can develop into functional endocrine cells both *in vivo* (28) and *in vitro* (29). Even using this tissue, however, the problem of hyperacute rejection (28), continuing controversy over transfer of endogenous porcine viruses to the human genome (30), and general public stigma over the use of animal organs for transplant have impeded the progress of this method as a clinical alternative.

There also exist several cell-based approaches to generate an abundant supply of islets or β-cells. The use of gene therapy and advanced transfection techniques to bioengineer suitable primary cells or cell lines has been used to create insulin-producing tissue (31, 32, 33). These studies, however, are plagued with problems relating to clinical applicability. For example, techniques for the reliable delivery of genes are lacking and sustained expression has been elusive (34). Furthermore, it will prove exceedingly difficult to re-create all of the required biological machinery to allow for insulin synthesis, release, and glucose responsiveness in a cell not predisposed to these processes (35). For these reasons, as well as issues involving immune rejection and the tumourogenic risk of transplanting cell lines, these approaches remain far from providing an answer to the problem of islet availability.

Among the most promising and most actively researched alternative islet sources is the use of embryonic or adult stem cells. The ability to isolate and expand progenitor cells that may subsequently be differentiated into pancreatic endocrine cells will represent a major advancement in the fields of islet transplantation and type 1 diabetes. Advantages to this approach include the possibility of propagating an unlimited number of cells that already possess the ability to become fully functioning endocrine tissue, as well as elimination of the aforementioned problems associated with xenotransplantation. Furthermore, the potential use of adult stem cells offers the advantage of an autologous model whereby a patient's own cells can be used, thereby circumventing immune rejection. Similarly, embryonic stem cells (ES cells) in an undifferentiated state have been proposed to be undefined immunologically and programmable by the recipient's own immune system as "self" tissue. The main drawback to the use of stem cells in islet transplantation at this time is simply that techniques to identify and subsequently differentiate stem cells to the islet endocrine phenotype are lacking. For this reason, the focus of this Introduction is to provide a comprehensive summary of research on both ES cells and adult stem cells that is centered towards the generation of an unlimited supply of insulin producing cells. Specific issues relating to the maintenance and differentiation of insulin producing cells from ES cell lines will be addressed. In addition, progress and considerations in the identification of adult islet precursor cells will be discussed in detail with a focus on potential locations for this elusive population.

I-D) Differentiation of embryonic stem cells into insulin-producing cells

The derivation of pluripotent stem cell lines from cells of the inner cell mass of a developing blastocyst has provided the potential for *in vitro* growth of virtually any somatic tissue type. Indeed, it has been shown that a variety of cellular phenotypes, including heart, brain, muscle, endocrine, and hematopoietic cells can arise from ES cell cultures. Protocols for establishment and maintenance of ES cell lines were created over 20 years ago using murine cells (36, 37), and many studies to this day still utilize mouse ES cells for the characterization of ES cell physiology and the development of differentiation protocols. In addition to significant advances with mouse ES cells, an important milestone was achieved in 1998 with the derivation of the first human ES cell lines by Thomson and co-workers (38). Although differences have been defined between mouse and human ES cells with respect to surface antigen expression, morphology, and culture requirements (39), both are derived in similar ways and exhibit similar differentiation characteristics. Culture conditions for both typically involve the maintenance of single, undifferentiated cells (characterized by expression of markers such as Oct4) followed by the formation of cellular aggregates (embryoid bodies), before the induction of cellular differentiation (38, 40). Initial protocols required the use of murine fibroblast feeder layers, complicating the issue of clinical feasibility in humans due to xenogeneic exposure, however newer methods require only conditioned media and defined growth factors (41), allowing the future possibility for transplantation to humans. Overall, the observation that mouse and human ES cells can be expanded indefinitely in an undifferentiated state and possess the inherent ability to develop into pancreatic islet endocrine cells suggests that these cells may be a potential source of transplantable tissue for type 1 diabetics. Furthermore, using techniques such as somatic cell nuclear transfer (42), ES cells could be created from a patient's own cells eliminating the possibility of immunorejection after islet differentiation and transplant.

One of the major limitations in ES cell research, however, is the inability to produce well-controlled, directed differentiation into specific tissue types. When cultured in suspension, these cells form embryoid bodies that have been shown to contain partially differentiated cells of all three embryonic germ layers (mesoderm, endoderm, ectoderm) (43). This heterogenous differentiation poses difficulties when attempting to create a large number of pancreatic islet cells. For this reason, specific culture conditions and growth factors have been utilized in an attempt to attain a more controlled, homogenous differentiation process. A study by Schuldiner and co-workers (44) analyzed the effects of certain growth factors on ES cell differentiation. While it was found that specific growth factors such as EGF and TGF-β could direct a percentage of cells towards a certain lineage, none were effective at producing homogenous cultures of a specific cellular phenotype. Importantly, it was also shown that the addition of nerve growth factor induced expression of some endodermal genes in these cultures, including the islet developmental transcription factor PDX-1. Although these results were promising, the problems with spontaneous differentiation were indicative of the difficulties still experienced today using ES cell lines.

One study that indicated the possible development of pancreatic islets from mouse ES cells and spurred a worldwide outbreak of subsequent research was that of McKay and co-workers (45). The protocol described in this study involved the production of an enriched cell population from murine ES embryoid bodies expressing the central nervous system precursor marker nestin through the use of serum free media, followed by expansion with basic fibroblast growth factor (bFGF). Differentiation involved withdrawal of bFGF and supplementation with B27 and nicotinamide to induce a pancreatic endocrine phenotype. Using these techniques, the authors reported the appearance of cells containing insulin as well as the other islet hormones glucagon, somatostatin, and pancreatic polypeptide. Expression of other markers of differentiated β-cells, such as PDX-1, glucose transporter-2 and islet amyloid polypeptide was also seen in these islet-like structures. Functional assessment showed some degree of glucose stimulated insulin secretion, although these cells contained far less insulin than a native β-cell and were not able to correct hyperglycemia when transplanted into diabetic mice. Interestingly, although a significant amount of research is now being based on this study, recent reports have questioned these findings (46). In particular, as the media used during the differentiation process was supplemented with extremely high concentrations of insulin, it has been proposed that insulin immunoreactivity in these cells is related to uptake from the media and not from endogenous synthesis. This proposal is supported by: i) lack of insulin mRNA by PCR, ii) absence of the proinsulin cleavage product c-peptide, iii) absence of secretory granules by electron microscopy, and iv) lack of activity from an introduced insulin promoter-driven GFP gene in subsequent experiments using the same protocol. Although controversy now exists over the significance of results reported by the McKay group, several labs continue to use similar techniques with improved analysis methods in an attempt to create a homogenous islet population from ES cells.

Since the McKay protocol for differentiation of nestin-positive mouse ES cells, a number of other groups have reported the derivation of insulin-containing cells from these cultures. A subsequent study, also utilizing murine ES cells, by Soria *et al.* (47) reported the generation of a relatively homogenous population of insulin-secreting cells using a cell trapping technique. In this study, a transfected neomycin gene under control of the insulin promoter was used to positively select insulin expressing ES cells and subsequently develop a clonal population. Grown under appropriate conditions, these cells were shown to contain significant amounts of insulin. Glucose induced insulin release, however, was abnormal and, although implantation led to transient correction of hyperglycemia in diabetic mice, this effect could not be maintained for extended periods. A study in 2001 by Assady *et al.* (48) was the first to report insulin production using human ES cells. Using the H9 human ES cell line, it was shown by immunostaining that a small percentage of differentiated embryoid body cells were positive for insulin and that insulin levels increased in the media compared to undifferentiated cultures. The expression of β-cell specific markers such as glucokinase and glucose transporter-2 was also observed in differentiated cultures although it was not proven whether cells expressing these markers were the same cells that stained positive for insulin. In addition to these earlier studies, several other groups have recently used specific culture supplements or gene transfection strategies to induce ES cells to insulin production. Hori *et al.* (49) used an inhibitor of the intracellular signaling molecule phosphoinositide 3-kinase to produce a population of insulin-containing cells from mouse ES cells. Insulin content in these cultures was reported to be about 30 times greater than that using the standard McKay protocol, although still only 10% of that seen in isolated islet preparations. Furthermore, aggregates were shown to be similar in morphology to islets, comprising predominately insulin-positive cells, some glucagon-positive cells, but no somatostatin or pancreatic polypeptide containing cells. These differentiated cells were also shown to improve the health of chemically induced diabetic mice, however their large size precluded the transplantation of a sufficient quantity to fully correct hyperglycemia. Another study by Wobus and co-workers (50) used electroporation to transfect mouse ES cells with the islet/β-cell developmental transcription factors PDX-1 and Pax4 in an attempt to induce pancreatic endocrine differentiation. They observed that Pax4 activation in nestin-positive embryoid bodies caused the induction of other transcription factors such as neurogenin 3, as well as an increase in insulin immunoreactivity (about 60% positive). Surprisingly, the authors do not address the possibility that increased insulin immunoreactivity in transfected cultures over wildtype cultures is due to increased cellular uptake from the media over controls as a result of membrane damage from electroporation. Furthermore, although the authors report rescue of experimentally induced diabetes in mice using these transfected cultures, the transplantation protocol used was questionable because animals were only followed for a relatively short time period of 14 days. Finally, a group from Belgium recently reported that the transition of mouse ES cells into insulin-containing cells using established protocols does not require the transcription factor HNF-6 (51). The authors used ES cells generated from HNF-6 knockout mice to reproduce the results obtained by McKay and others. The significance of this study is that normal β-cell development is absolutely dependent on expression of HNF-6, showing that the mechanism of ES cell differentiation in these experiments differs from that of natural islet formation during development.

Overall, the progress toward generating clinically transplantable islets from ES cells, although promising, has been hampered by fundamental biological hurdles involving culture conditions and control of differentiation. Regulatory and ethical issues, which are beyond the scope of this thesis but are comprehensively reviewed in other manuscripts (52, 53), also provide an obstacle for the clinical use of human ES cells. It should also be noted that the creation of a homogenous insulin producing, glucose-responsive cell population from ES cells may not be sufficient to provide a source for transplantation. The need to create a fully functional islet structure consisting of all four endocrine cell types should be recognized by researchers and this should be the ultimate goal of these experiments.

I-E) Identification of the elusive adult pancreatic stem cell

Another possible solution to the problem of islet supply is the use of stem cells derived from adult tissues. The potential use of adult stem cells offers the advantage of an autologous model in which a patient's own cells could be used, thereby eliminating the problem of graft rejection. The concept that stem cells exist in adult tissues was proposed years ago, and they have subsequently been identified in a diverse range of tissues, including liver, intestine, and skin. Recent advancements in adult stem cell research include the isolation and *in vitro* manipulation of neural (54, 55), hematopoietic (56), and muscular (57) progenitor cells. The identification and exploitation of a pancreatic stem cell or precursor cell, using similar techniques, would represent a significant advancement for cell replacement therapy for type 1 diabetes.

Islet neogenesis and transplantation of adult pancreatic stem cells is a concept that has attracted significant research attention. Figure 1-2 summarizes the possible cell sources for the formation of new islet endocrine cells in the adult pancreas. Evidence of continuous cell turnover in other organ systems (e.g. blood, intestine) throughout life suggests that the proportion of endocrine cells in the pancreas may also undergo dynamic changes in response to growth, development, and conditions such as pregnancy or obesity. A study by Finegood *et al.* (58) proposed a mathematical model to estimate the dynamics of β-cell turnover in the pancreas. According to this model, a balance is maintained in the pancreas between the processes of cell division, growth, and death.

Furthermore, the lifespan of an average β-cell was proposed to be variable depending on replication rate but most likely from 1-3 months. This data would suggest that a significant amount of cell turnover in the pancreas occurs throughout life. Furthermore, studies involving experimentally induced pancreatic damage in animal models also support the idea of a stem cell population. Partial pancreatectomy in rats has been shown to result in islet regeneration and compensation for decreased circulating insulin levels (59). Other modes of damage such as chemical destruction of islets using alloxan or streptozotocin (60, 61), cellophane wrapping (62), ductal ligation (63), and exposure to transient hyperglycemia (64, 65) have also been proven to result in pancreatic endocrine cell regeneration. Although evidence exists that β-cells themselves may be induced to replicate under certain conditions (66, 67), the level of islet turnover apparent in the pancreas, as well as the fact that terminally differentiated cells do not normally undergo active proliferation, suggests that islet neogenesis from a pancreatic precursor cell plays a significant role in islet turnover.

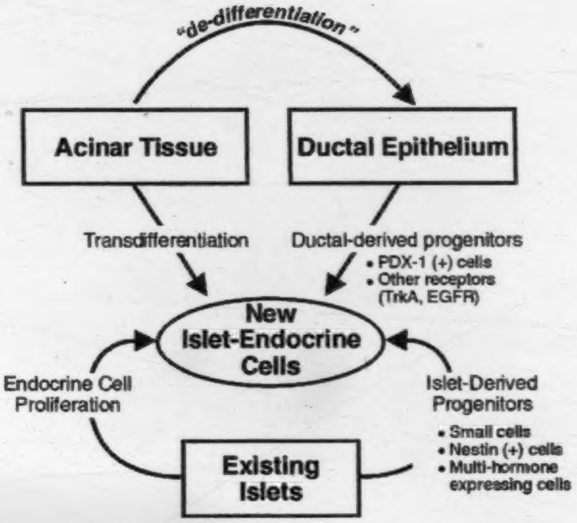
"de-differentiation"
Acinar Tissue
Ductal Epithelium
Transdifferentiation
Ductal-derived progenitors
• PDX-1 (+) cells
• Other receptors (TrkA, EGFR)
New Islet-Endocrine Cells
Endocrine Cell Proliferation
Islet-Derived Progenitors
• Small cells
• Nestin (+) cells
• Multi-hormone expressing cells
Existing Islets

Figure 1-2: Overview of the possible sources of new islet cells in the adult pancreas. Dynamic changes in the exocrine (acinar), ductal, and endocrine compartments allow continuous islet turnover throughout life. Direct transdifferentiation or de-differentiation of acinar cells to ductal phenotype may play a role in islet neogenesis. The role of ductal epithelium has been well established in the creation of new islets however it has not been elucidated whether all ductal cells possess this ability or whether specific sub-populations (defined by protein/receptor expression) of islet progenitor cells exist within the ducts. Finally, islets themselves most likely also contribute to maintenance of endocrine mass through either proliferation of existing endocrine cells and/or differentiation of islet-derived progenitors.

There is a substantial amount of evidence to support the hypothesis that islet neogenesis in the mature pancreas occurs via cells in or associated with the ductal epithelium. Ductal cells comprise about 5-10% of the normal pancreas and form the transport network for release of digestive enzymes into the gut. These cells are characteristically simple, undifferentiated, and hence lack specific identification markers such as those used for endocrine or exocrine cells. Despite this, ductal cells have been phenotypically defined in human and animal models through expression patterns of specific cytokeratin intermediate filament proteins. Ductal cells in both rat and human pancreas as well as pancreatic cultures have been shown to express a variety of cytokeratins; most prominently CK7 and CK19, in a reliable pattern (68, 69, 70). More recently, the lectin cell surface protein Dolichus Biflorus Agglutinin (DBA) was also proposed as a marker for ductal epithelium and was used to isolate a population of these cells from a heterogenous pancreatic culture (71).

Evidence for elucidating the mechanisms of islet neogenesis in the adult pancreas may result from the study of embryonic and fetal pancreatic development. It is known that early pancreatic development from the endodermal bud progresses via “branching morphogenesis” of cytokeratin expressing ductal structures (72, 73). Cells within these structures eventually lose expression of cytokeratins and develop into both the endocrine and exocrine compartments of the pancreas (72). In the adult pancreas, individual ß-cells as well as intact islets have been observed in close association with cytokeratin-positive ductal epithelium (74, 75). Transitional cells, expressing both insulin and CK19 have also been described in adult human pancreatic sections (75). These data provide evidence that pancreatic stem cells may be ductal in nature or reside in close association with ductal epithelium.

Experimental evidence, both *in vivo* and *in vitro*, exists to support the theory that islet neogenesis in the mature pancreas occurs from ductal cells. In 1991, it was shown that the co-transplantation of adult rat pancreatic epithelium and fetal-derived mesenchyme into the epididymal fat pad of rats resulted in the appearance of cells expressing both insulin and glucagon (76). This study provided evidence for the widely held belief that factors released from surrounding mesenchymal tissues can induce pancreatic islet differentiation. As discussed earlier, several models of pancreatic damage have also shown the regeneration of islets *in vivo* from ductal cells. In particular, Wang *et al.* (63) reported that ligation of the tail of the pancreas in rats resulted in a ductal to endocrine transition via proliferation and subsequent differentiation of cytokeratin-positive cells.

Specific culture conditions and *in vitro* manipulation of pancreatic ductal cells has also been used to demonstrate that ductal tissue contains islet precursor cells. Bonner-Weir *et al.* (77) cultured human ductal cells as a monolayer overlaid with Matrigel^{TM}, and observed the growth of "islet buds" containing CK19 expressing as well as insulin-positive cells. Moreover, these cultures demonstrated increased insulin content and some degree of glucose-induced insulin secretion. In addition, a more recent study from Heimberg and co-workers (78) showed that viral transduction of the early islet developmental transcription factor neurogenin 3 in human ductal cell cultures could initiate differentiation to ß-cell phenotype. Although these studies show convincing evidence that ductal cells contribute to islet neogenesis in the adult, the low proportions of differentiating cells suggests that either the methods are as yet inefficient, or that only a specific sub-population of ductal cells are true islet progenitors.

Several lines of evidence also suggest that a subpopulation of cells exist within the pancreatic ducts that may be endocrine precursors. For example, ductal cells have been shown to exhibit plasticity and are capable of expressing non-wildtype proteins under abnormal conditions. The expression of PDX-1, for example, has been documented in pancreatic ductal cells. PDX-1 is a homeobox transcription factor expressed in all pancreas-dedicated cells of the endoderm during early development (79). Over the course of organogenesis, however, expression is gradually lost until only mature ß-cells of the adult pancreas express PDX-1 (80). It has been shown that PDX-1 is absolutely necessary for proper pancreas formation, as mice that are homozygous for the null mutation are born without a pancreas and die shortly thereafter (81). PDX-1 has been demonstrated to be re-expressed in ductal cells of the mature pancreas under certain conditions. Leach and co-workers (82) demonstrated the increased expression of PDX-1 in pre-malignant ductal epithelium in the pancreas of transgenic mice overexpressing transforming growth factor-alpha. Another study reported the increased expression of PDX-1 in rat ductal cells after partial pancreatectomy (83). Furthermore, Heimberg *et al.* (84) showed the presence of PDX-1 in a significant proportion of human pancreatic ducts, although phosphorylation patterns and complex formation was different than in mature ß-cells. Pancreatic ductal cells in culture have also been shown to re-express PDX-1, as they do transiently during embryonic development. Non-endocrine pancreatic cultures derived from both rodent and human pancreas, consisting of primarily ductal cells, showed increased levels of PDX-1 expression (85, 86). Results from our lab also suggest that the proportion of ductal cells expressing PDX-1 after several days culture is donor-age dependent, with an increase seen in young donors (<25 yrs) but not in older donors (unpublished observations). There is also evidence to indicate that PDX-1 plays a role in islet neogenesis in the mature pancreas. A recent study has shown that viral-mediated delivery of the PDX-1 gene into mouse pancreas induced ductal cell proliferation and subsequent ß-cell neogenesis (87). Moreover, as will be discussed in the next section, ectopic expression of PDX-1 in non-pancreatic tissues can also induce differentiation into islet-like cells (88, 89). This data provide evidence that ductal cells in the mature pancreas that express PDX-1 may potentially represent islet precursor cells. Interestingly, a recent study by Melton and co-workers (90) utilized a Cre-LoxP cell lineage tracing system to show that, although all pancreatic cells express PDX-1 very early in development, cells destined to become ducts are distinct from those destined for endocrine fate. Although this would seem to imply that endocrine cells do not arise from ductal cells in the mature pancreas, it can be argued that the ability of mature ductal cells to re-express PDX-1 indicates a reversion back to early embryonic phenotype and possible lineage-switching capabilities.

Although substantial evidence suggests that islet progenitor or stem cells exist within the pancreatic ducts, the identification and isolation of these has remained elusive. As noted above, ductal cells that express PDX-1 are one possible candidate. In order to further narrow the search for these precursors, it may be useful to look to other, more extensively characterized tissue model systems. Adult stem cells have been identified in tissues such as muscle (57), CNS (54, 55), and blood (56) and have been isolated based on expression of characteristic cell surface proteins. Trk-A, the receptor for nerve growth factor (NGF), for example, has been shown to be expressed in neurosphere-derived stem cells and to be an effector of neural differentiation (91). This receptor has also been shown to be expressed by hepatic stellate cells (92), which are involved in liver remodeling and regeneration. Interestingly, Trk-A expression has also been described in pancreatic cells. Miralles and co-workers (93) demonstrated its presence on pancreatic exocrine AR42J cells, and other studies have shown the ability of this cell line to differentiate into insulin-producing cells (94). Moreover, it is also known that Trk-A is expressed on ductal-epithelial cells of the embryonic and fetal rat pancreas at specific developmental stages (95), and that islet morphogenesis is reduced in the presence of agents that reduce tyrosine kinase activity in this receptor (96). In the adult rat pancreas, islet ß-cells express NGF (97), and Teitelman *et al.* (98) showed that expression is upregulated in response to pancreatic damage by streptozotocin treatment. This suggests that cells expressing Trk-A in the mature pancreas may play a role in islet regeneration. It is yet to be demonstrated whether a subpopulation of Trk-A-positive cells can be isolated from the adult pancreas and what significance they have in islet neogenesis.

Other receptors or cell surface proteins may also be potential markers to identify and isolate pancreatic stem cells. The hyaluronan receptor CD44 (HCAM) is a glycoprotein molecule involved in cell attachment and cell-cell interactions (99). This protein is expressed in normal pancreatic ducts (100, 101), and is upregulated in metaplastic ductal cell adenocarcinomas (102). Furthermore, it has been proposed that CD44 expression in hyperplastic ductal clusters is an indicator of de-differentiation. Data from our lab (unpublished observations), also suggest that human ductal cells can initiate CD44 expression in culture. CD44 expressing ductal cells, therefore, are another possibility of a mature pancreatic progenitor cell. This receptor, however, is also expressed in non-pancreatic cells (e.g. lymphocytes, mesenchymal cells) that may be found in the pancreas and, as such, caution should be used when investigating its role in islet formation. Hu *et al.* (103) recently reported the isolation of a proliferating mesenchymal stem cell population from human fetal pancreas that expresses CD44 and can be differentiated into osteogenic, chondrogenic, and adipogenic lineages. It remains to be seen whether these non-endodermal cells are capable of differentiation to a pancreatic lineage.

Although pancreatic ductal cells are a probable source for adult pancreatic stem cells, extensive research has been carried out to ascertain if other pancreatic cell types play a role in the birth of new islets. Other potential stem cell markers have been identified in the pancreas, and recent widespread interest in the phenomenon of transdifferentiation of specialized cell types has opened new possibilities for involvement of other cell types. Some non-ductal pancreatic candidates for islet precursors include pancreatic small cells (104), exocrine/acinar cells (105), and cells that express the protein nestin (106).

Nestin is an intermediate filament protein involved in cytoskeletal formation as well as cellular re-arrangement to facilitate processes characteristic of undifferentiated cells, such as migration and mitosis (107, 108). It has also been shown that nestin can be used to identify multipotent cells in the adult CNS that are capable of differentiating along several neural lineages (109-112). In the pancreas, nestin-positive cells have been identified in murine (113), rat (106, 114), and human (115) islets and evidence suggests that these cells can be induced to differentiate into islet endocrine cells (106, 115). Controversy does exist, however, over the role of nestin-positive cells in islet neogenesis. Several studies report that nestin is not expressed in endodermal pancreatic cells, but rather in cells of mesenchymal origin (114, 116, 117). In contrast, other studies demonstrate nestin expression in rat pancreatic cells and suggest that these are islet precursors (106). Results from our lab (118) indicate that nestin is expressed heterogeneously in both pancreatic and mesenchymal (ie. vasculature) tissues and that the majority of these cells are likely not islet precursors. For these reasons, although it cannot be ruled out that nestin may play a functional role in islet differentiation, it is most likely not a suitable marker on its own for pancreatic stem cells.

The potential role of pancreatic exocrine cells in islet neogenesis is also a matter of debate. Several studies have reported the *in vitro* transdifferentiation of exocrine cells into ductal-like cells that may then be capable of differentiation into endocrine cells. Early studies by Delisle and Logsdon (119) and Hall and Lemoine (120) proposed that acinar cells in long-term culture lose expression of digestive enzymes such as amylase and begin to express cytokeratins and mucin antigens. Later studies also reported this phenotypic transition in cultures of rat (85), hamster (121), and human (86, 122) non-endocrine pancreatic tissue. Evidence to support the proposal for transdifferentiation, however, is lacking in all of these studies and alternative explanations may be more attractive. For example, selective cell death of exocrine cells and survival of the more robust ductal cells may be the predominant mechanism at work in these cases. Due to the stresses of collagenase digestion and purification during islet isolation, combined with the fact that primary pancreatic cells do not normally flourish in culture, a substantial number of exocrine cells (the predominant cell type) will die in the first days of culture. In these studies, the observance that resulting ductal cultures re-express proteins such as PDX-1 (85, 86) is interpreted as evidence that exocrine cells, via a ductal intermediate, may act as islet progenitors. Strong evidence for exocrine to ductal cell transdifferentiation, however, must be provided before this can be considered a candidate pathway for islet neogenesis. *In vivo* studies have also proposed ß-cell neogenesis via exocrine to ductal transdifferentiation in an experimentally damaged pancreas model. Rooman *et al.* (105)

infused gastrin into pancreatic duct ligated rats and observed a doubling in ß-cell mass in the ligated vs. unligated portion. This was interpreted as evidence that exocrine cells under insult can convert to ß-cell phenotype. The fact, however, that the weight of the ligated half of the pancreas was significantly lower than the unligated half suggests again that exocrine tissue may not survive, leaving ductal enriched tissue for subsequent differentiation into islet tissue. Although exocrine to ductal transdifferentiation has never been proven definitively, it also cannot be ruled out as a contributor to islet neogenesis in the mature pancreas. Arguments both for and against pancreatic transdifferentiation have been reviewed extensively (123, 124), and improved cell "tracking" techniques or clonal analysis will eventually elucidate the contribution of this mechanism.

Finally, there also exists the possibility that islets themselves contain progenitor cells that allow their prolonged survival and continual cellular turnover. It is known that, along with the four endocrine cell types, islets contain other non-endocrine cells. For example, ductal-like cells, expressing cytokeratins, can often be observed in the islet periphery, and these cells may play a significant role in islet cell neogenesis. Furthermore, the existence of "small cells" within pancreatic islets has been reported (104). These cells are described as immature, small in size, and representing about 1% of islet cells. These small cells also express all four endocrine hormones, as well as PDX-1, synaptophysin, alpha-fetaprotein, and Bcl-2, and are able to secrete insulin in response to glucose. Proliferation and differentiation, however, have yet to be demonstrated in these cells and their role in islet formation is as yet uncertain. Guz and co-workers (125) also report the existence of a progenitor cell in the islet itself. They observed cells co-expressing both insulin and somatostatin in islets of pancreases exposed to streptozotocin and thereby propose these to be repopulating cells in the process of ß-cell differentiation. Although the concept of an intra-islet precursor is attractive, it remains a question whether these structures have the capacity for the extensive cell replication required for self-sustenance.

I-F) Existence of non-pancreatic islet progenitor cells

Given recent experimental findings demonstrating evidence for the existence of multipotent adult stem cells that can give rise to several different tissue types (125), it is reasonable to hypothesize that new islet cells may be derived from non-pancreatic sources. Figure 1-3 summarizes the potential mechanisms for islet neogenesis from extra-pancreatic sources. Firstly is the potential to induce cells of another organ system to differentiate into islets via gene therapy or the induction of cellular transdifferentiation using other manipulations. More recently, is the concept that self-renewing, multipotential cells in adult tissues are independently capable of differentiation into islets. This latter proposal represents a change from the view that every organ has its own discrete population of committed progenitor cells, and that cell division and differentiation act to gradually narrow a cell's fate, eventually resulting in unipotency, without the possibility of reversion or lineage-switching. In contrast, there is also the concept that a universal, primitive stem cell persists to adulthood that could give rise to all somatic cell types. Moreover, if true, these would have much the same potential as embryonic stem cells, without the ethical and legal issues. Several reviews (127, 128) have described adult stem cell plasticity and interconversion of tissue types. In this section, the experimental evidence for pancreatic islet cell formation from other adult tissues will be addressed, with attention given to maintaining the distinction between cell transdifferentiation and true adult stem cell differentiation.

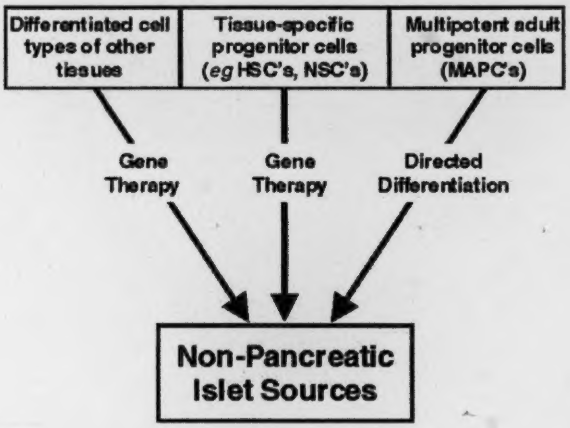
Differentiated cell types of other tissues
Tissue-specific progenitor cells (*eg* HSC's, NSC's)
Multipotent adult progenitor cells (MAPC's)
Gene Therapy
Gene Therapy
Directed Differentiation
Non-Pancreatic Islet Sources

Figure 1-3: Representation of possible extra-pancreatic sources for islet neogenesis and mechanisms by which this could be achieved. Differentiated cell types of other tissues can be manipulated using gene therapy to express insulin and other islet cell markers. Similarily, tissue-specific committed progenitor cells (e.g. hepatic oval cells) have also been genetically modified to adopt a β-cell-like phenotype. Finally, evidence suggests that a population of multipotent stem cells may persist to adulthood that could be isolated and induced to islet endocrine differentiation.

Several studies have proposed that terminally differentiated cells of another tissue type can be induced into a ß-cell phenotype, including the production and secretion of insulin. Although this does not represent the identification of an islet progenitor cell, success in this regard would be significant for the treatment of type 1 diabetes.

Conversion of intestinal, hepatic, or other cell types requires some form of genetic manipulation. Cheung *et al.* (30) were successful in engineering a gut K-cell line to exhibit glucose-stimulated insulin release, and another study was able to induce insulin expression in hepatocyte cell lines, myoblasts, and fibroblasts (31). As mentioned earlier, however, these types of studies are limited by the inefficiency and instability of gene transfection techniques, as well as the extreme difficulty in re-creating the complex biological ß-cell machinery.

Rather than attempting to specifically engineer a mature islet endocrine cell, it may be more reasonable to use gene therapy to induce islet differentiation through expression of transient developmental transcription factors. It is known that ß-cell development occurs via a cascade of transcription factors, some of which are functional in the mature ß-cell and some of which act only over a short period to induce other downstream genes in the pathway. In this regard, the fact that current transfection/transduction techniques are unstable would not be detrimental so long as the developmental pathway could be commenced by transient expression of upstream initiators. To this end, other studies have examined the targeted expression of developmental transcription factors to non-pancreatic cells in an attempt to induce islet differentiation. Yoshida and co-workers (129) used plasmid lipofection to deliver the PDX-1 gene into an intestinal derived cell line. Subsequently, they observed the expression of several other downstream ß-cell specific genes including amylin, Nkx6.1, and glucokinase. They also demonstrated insulin expression and secretion upon transplantation in a rat model, although these cells were not glucose responsive. Another study, using transgenic Xenopus tadpoles, reported the transdifferentiation of developing liver cells to pancreatic endocrine and exocrine cells through directed expression of PDX-1 (130). The authors showed that transient expression is sufficient to create ectopic pancreatic tissue that persists after loss of the transgene. A similar study by Ferber *et al.* (89) directed PDX-1 expression to the livers of adult mice and showed an induction of insulin production in sufficient amounts to correct hyperglycemia in chemically induced diabetic animals. These studies demonstrate that some degree of transdifferentiation between tissue types can be achieved experimentally. This may be of use in the treatment of diabetes in the future, although it by no means implies that transdifferentiation occurs naturally in an adult organism.

By an entirely different mechanism, it is possible that non-pancreatic adult stem cells exist that are capable of giving rise to pancreatic islets. These may be stem cells specific to other tissues or "universal" stem cell populations persisting from early embryogenesis. It has been shown that, early in development, committed progenitor cells exist that will form the mature organ systems. This fate determination depends on specific cell signaling and gene expression and can be experimentally altered. For example, activation of the gene Ptf1a is critical in the decision of putative pancreatic progenitors in the endodermal bud to form pancreatic cells as opposed to intestinal progenitors (131). In the adult, hepatic oval stem cells have shown the ability *in vitro* to express islet cell characteristics, including insulin secretion in response to glucose (132), indicating a high degree of plasticity. Furthermore, although the differentiation of pancreatic islet cells from adult neural stem cells has not yet been demonstrated, the similarities in developmental pathways and gene expression between islets and other neuroendocrine cells suggests that this conversion may be possible.

Finally, there is the potential that non-tissue specific multipotential cells persist to adulthood that could give rise to all somatic cell types. Jiang and co-workers (126) demonstrated the existence of these cells (named multipotent adult progenitor cells or MAPCs) in bone marrow of mice and rats. These cells exhibited extensive proliferation, and could be differentiated into cells with mesodermal, ectodermal, and endodermal characteristics. This has raised the idea that islet neogenesis could be achieved using stem cells derived from the bone marrow and recent evidence has been shown to support this theory. Ianus *et al.* (132) performed an experiment in mice whereby male-derived bone marrow cells, engineered to express GFP upon transcription of the insulin gene, were transplanted into female recipients. At 4-6 weeks after transplantation, GFP-positive cells were observed in pancreatic islets that also expressed the Y chromosome, confirming a donor origin. Upon subsequent isolation, these cells were reported to express insulin, as well as have other ß-cell characteristics, and were shown to secrete insulin in response to increased glucose concentrations. The fact that donor-derived cells were observed in the islets, and did exhibit transcriptional activity from the insulin gene, shows that non-pancreatic adult stem cells may contribute to islet neogenesis in the mature pancreas. Reproduction of these results along with further analysis will elucidate whether bone marrow derived stem cells can in fact undergo ß-cell differentiation and are a potential source of islets for transplantation.

I-G) SUMMARY

With the recent advancements in islet transplantation, cell replacement therapy is now a real alternative for the treatment of type 1 diabetes. Current limitations to this approach include the lack of a sufficient source of insulin-producing tissue in addition to that available from cadaveric donors. The most promising solution to this problem is the creation of pancreatic islets from embryonic or adult stem cells. The use of ES cells has received much attention, and preliminary results indicated that islet-like structures could be produced from these primitive cultures. However, limitations including difficulty in controlling differentiation of ES cells have hampered progress, and researchers must further understand fundamental ES cell biology before attempts can be made toward clinical application. Moreover, in relation to type 1 diabetes these problems are further amplified by the need to not only create one cell type, but to create a fully functioning, multicellular islet containing all four endocrine cell types. This goal may prove exceedingly difficult *in vitro* and may necessitate the use of *in vivo* animal models to achieve a suitable environment for islet differentiation.

Islet neogenesis has been well documented in the adult pancreas, and the existence of an adult pancreatic precursor cell is almost certain. As yet, however, these cells have remained elusive, and debate continues over their identification and significance. The greatest body of evidence suggests that these progenitors are associated with pancreatic ductal epithelium, although it is not known whether all ductal cells possess the capability for islet neogenesis or if subpopulations exist expressly for this function. The identification of a pancreatic stem cell will represent a significant advancement, and in this regard rigorous assessment will be necessary to confirm its identity. For example, these cells should be capable of self-renewal, differentiation to all endocrine cell types, and migration to form intact islet structures. Clonal analysis will undoubtedly be necessary to confirm the potential of these cells. Alternatively, the possibility exists that more committed, unipotent islet precursor cells exist in the adult pancreas. In this case, it must be assessed whether these are a static population, capable of self-renewal, or produced from another cell type (ie. transdifferentiation or de-differentiation). In addition, the development of protocols for the reliable *in vitro* differentiation of these cells will be necessary for them to be useful in cell replacement therapy.

Finally, there is the possibility that non-pancreatic cells may contribute to islet neogenesis in the adult. Some evidence exists that closely related tissues, such as liver and intestine, contain cells capable of "switching" to pancreatic phenotype under the appropriate genetic direction. Recent studies have also proposed the existence of multipotent adult stem cells that may give rise to multiple tissue types, and could be involved in islet neogenesis. This emerging area, while promising, requires further research and strict experimental protocols to rule out events such as cell fusion and ensure the distinction between host and donor cells. Furthermore, it remains to be seen whether these circulating stem cells contribute in meaningful amounts to specific tissues or are an anomaly with no significant functional value.

In summary, although obstacles exist to the application of both ES and adult stem cells in islet transplantation protocols, these represent the most promising alternative for creating an abundant source of transplantable islets. As newer methods are developed for the identification and manipulation of these cells, understanding will increase as to how to use them to replace lost or damaged tissues. The creation of pancreatic islets from stem cells in the future has the potential to benefit millions with type 1 diabetes, and alleviate much of the cost and suffering associated with this disease.

I-H) OBJECTIVES AND GENERAL OUTLINE OF THESIS

The primary objective of this thesis is to identify and induce differentiation from an islet progenitor cell derived from the adult pancreas. A large body of evidence suggests that these progenitor cells are associated with pancreatic ductal epithelium, and therefore the focus of this work was placed on studying ductal cell populations from the human pancreas in an attempt to elucidate a model for islet differentiation and to provide further evidence for a ductal progenitor sub-population. If this can be accomplished, it is foreseeable that a clinical application can be developed for the use of non-endocrine pancreatic tissue that is normally discarded after islet purification.

Chapter 2 of this thesis provides a starting point for the remaining studies in that we have elucidated a model for the derivation of a relatively pure ductal cell population from non-endocrine human pancreatic digest. In this project, we have built upon previous studies and developed a serum-free culture model that allows selective cell death of acinar cells and preferential survival of the ductal component. These ductal populations are necessary to carry out the remaining studies in this thesis, which build upon this initial protocol in an attempt to induce islet differentiation. Furthermore, in this chapter, we show that the developmental transcription factor PDX-1 can be induced in a population of ductal cells after culture, and that the amount of PDX-1 expression is donor-age specific. This result suggests that young donors may provide advantages in the form of increased cell plasticity and differentiation potential.

In the next several chapters, we provide evidence that ductal cells can be induced to islet lineage, as well as examine several candidate ductal subpopulations for progenitor potential. In Chapter 3 a genetic approach was taken whereby human ductal cells were transduced with the developmental transcription factor neurogenin 3 to drive the β-cell differentiation process. It has been previously shown that this manipulation can result in limited differentiation to insulin-producing phenotype *in vitro*, however we were not able to reproduce these results in our lab. Instead, only partial differentiation was seen *in vitro* and full β-cell differentiation was only observed in a novel *in vivo* protocol; in which transduced cells were transplanted into the pancreas of chemically induced diabetic animals. This study showed that with the proper manipulation adult human ductal cells can undergo islet neogenesis, although, as in other studies of this kind, the limited numbers of differentiating ductal cells suggests that either the process is inefficient or that only a sub-population of ductal cells are islet progenitors.

Chapters 4 and 5 are focused on the analysis of specific pancreatic cell markers/receptors as indicators of an islet progenitor phenotype. In Chapter 4, two ductal sub-populations were identified and analyzed, based on the expression of the cellular receptors CD44 and Trk-A. It was shown that a significant number of human ductal cells in culture express these receptors and that these populations express other genes that are involved in islet neogenesis. Furthermore, it was shown that human β-cells express the Trk-A receptor, as the vast majority of insulin expression in these preparations was in the Trk-A positive cell populations. Chapter 5 examines the expression pattern of another proposed islet precursor marker, nestin, in human pancreatic tissue and in clinical islet graft samples. It is concluded that the heterogenous pattern of nestin expression in acinar, ductal, islet, and menchymal cells precludes its use as an islet progenitor marker on its own, although it cannot be ruled out that nestin may be expressed in progenitor cells.

Finally, Chapter 6 of this thesis shifts focus to the analysis of human clinical islet grafts, and whether the presence of ductal cells in these grafts has an effect on patient metabolic outcome. In this study, more than 80 clinically transplanted islet grafts were assessed over a four year period for phenotypic composition, mass, insulin content, and function. These results were then used in an attempt to define a good predictor of transplant outcome to improve transplant success rates. Although factors such as *in vitro* function did not correlate with patient outcome, an important finding was made in that patients receiving a greater number of ductal cells had better metabolic function in the long term (>1 year post-transplant) indicating that islet neogenesis from transplanted progenitor cells may prolong graft function. This finding provides further evidence that ductal cells are in fact these progenitors, and that the presence of these cells in clinical islet grafts may be beneficial to transplant outcome.

In summary, this thesis advances the understanding of adult pancreatic ductal cells as a possible alternative source of islets for the cell-based treatment of type 1 diabetes. Several novel discoveries suggest that these cells have a substantial level of plasticity, and may be induced to islet phenotype. Furthermore, these studies suggest the need for a more detailed analysis of ductal cells as it is likely that only a sub-population of these cells are in fact islet progenitors. If these sub-populations can be identified, isolated, and expanded, the process of islet neogenesis will be much more efficient, and a clinically relevant number of insulin-producing cells may be produced for transplantation.

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CHAPTER II

ENRICHED HUMAN PANCREATIC DUCTAL CULTURES OBTAINED FROM SELECTIVE DEATH OF ACINAR CELLS EXPRESS PANCREATIC AND DUODENAL HOMEOBOX GENE-1 (PDX-1) AGE DEPENDENTLY*

II-A) INTRODUCTION

With recent advancements in islet isolation and immunosuppresive therapy (1-3), islet transplantation is now an effective treatment for certain individuals with type 1 diabetes. Limiting this effectiveness, however, is the fact that it presently requires at least 10, 000 islet equivalents/kg to consistently achieve insulin independence (1-3) necessitating the use of 2-3 donor organs for each recipient. For this reason, there exists the need for an increased supply of functional insulin-producing tissue in order to make islet transplantation a widespread treatment for patients with type 1 diabetes. Various alternative sources of insulin-producing tissue have been proposed including porcine tissue (4), engineered beta-cell lines (5), embryonic stem cells (6, 7), and pancreatic ductal tissue (8, 9), each with its own limitations. It is believed that, in the adult pancreas, cells of the ductal epithelium have the potential for differentiation to endocrine cells and may be one source of islet neogenesis throughout life (8, 9). Bonner-Weir *et al.* reported the development of human islets from a ductal-enriched population *in vitro* (10), showing this to be an effective source, however to date a clinically significant number of islets has not been produced by this or similar methods.

** A version of this chapter has been accepted for publication in "The Reviews of Diabetic Studies"*

The derivation of a ductal cell population through tissue culture of digested non-endocrine pancreatic tissue has been accomplished in several *in vitro* models (11-14). Culture of human and rat exocrine-enriched cell preparations has been proposed to result in a conversion from a primarily amylase-expressing cell population into a cell population that no longer expresses amylase but rather the ductal markers cytokeratin 7 and 19 (11-15). In other experiments, these ductal-like cells have been shown to be capable of expressing early endocrine markers (14, 16) or to have the capability to differentiate into rat β-cells *in vivo* (17). These results suggest that ductal cell populations could potentially provide an abundant source of islets for transplant to type 1 diabetic patients.

Although transdifferentiation between phenotypes has been suggested as the mechanism for the derivation of these ductal cell populations, it has not been proven whether this actually occurs. We hypothesized that these cultures of predominately ductal cells arise from selective cell death of the exocrine component and preferential survival of the ductal population during tissue culture. To test this hypothesis, human pancreatic cultures were analyzed for overall cell survival, levels of apoptosis, and the presence of transitional cells (ie. expressing both acinar and ductal markers) indicating a phenotypic intermediate between ductal and acinar. In addition, previous studies examining the preparation of enriched ductal populations have utilized serum-supplemented media (11-16) and it has been reported that for rat exocrine/ductal cultures to survive, serum must be added to the culture medium (14). However, in order for islets created in the future from these cultures to be used clinically to treat diabetes, a culture environment free of xenoproteins will be desirable. Thus, in the present study, both serum-supplemented and novel, serum-free formulations were tested for efficiency in deriving an enriched population of ductal cells. As it has been proposed that human pancreatic ductal cultures obtained in this fashion may be used to create an abundant source of islets for transplantation via differentiation of endocrine progenitors, preparations were also analyzed for expression of genes involved in islet development and mature islet function.

The homeodomain transcription factor Pancreatic and Duodenal Homeobox Gene-1 (PDX-1) is expressed in mature β-cells ubiquitously (18) and has been proposed to play a role in islet development both during embryonic organogenesis (19, 20) and to affect islet turnover in the postnatal pancreas (21, 22). Furthermore, other studies have shown that ectopic expression of PDX-1 in non-pancreatic cells is sufficient to induce differentiation to an insulin-producing phenotype (23, 24). Since PDX-1 expression has been previously reported in human ductal cell cultures (14, 16) we assessed the levels of PDX-1 expression quantitatively with the hypothesis that cultures containing higher numbers of PDX-1-positive ductal cells may exhibit greater plasticity and have a higher potential for islet neogenesis.

II-B) MATERIALS AND METHODS

1. Tissue preparation and culture

Human donor pancreases were removed from cadaveric donors who had previously given informed research consent and processed according to the protocols described by this laboratory (1, 25). Briefly, organs were cold stored in University of Wisconsin solution and perfused via the duct with the enzyme solution Liberase (Roche, Indianapolis, USA). Once digested, islets were purified on continuous Ficoll gradients using the refrigerated Cobe 2991 (COBE BCT inc., Lakewood, USA). Immediately following islet purification, the predominately acinar cell fraction was collected from the Cobe bag and washed three times with Hanks balanced salt solution (HBSS; Sigma-Aldrich, Oakville, Canada) supplemented with 0.5% bovine serum albumin (BSA; fraction V, Sigma). After dithizone staining of representative samples to assess islet content, tissue was cultured in suspension with approximately 500 μl "pack tissue volume" per non-treated 15 cm plate (Fisher Scientific, Edmonton, Canada) in RPMI 1640 medium (Gibco/Invitrogen, Burlington, Canada) supplemented with either: i) 10% fetal calf serum (FCS), ii) 1% insulin-transferrin-selenium (ITS)/0.5% bovine serum albumin (BSA), or iii) 1% ITS/0.5%human serum albumin (HSA) (all supplements/sera from Sigma). Media changes were performed at day 1 and day 3 post-culture. Protocols used in this study were approved by the Research Ethics Board of the University of Alberta.

2. Characterization of Cell Preparations

After isolation, as well as 4 day tissue culture, cell preparations were assessed for cellular insulin and DNA content as well as cell composition according to methods previously described (26). To determine cellular composition, aggregates were dissociated into single cell suspensions to facilitate quantification of immunostaining. Single cell suspensions were obtained by mechanical disruption by pipetting at 37°C in Ca^{2+}-free media supplemented with 1 mM EGTA and 0.5% BSA for 7 minutes before addition of trypsin (25 μg/ml) and DNAse (4 μg/ml) and further pipetting for 4 minutes. Cells were then allowed to adhere to histobond slides (Marienfeld, Germany) and fixed in Bouin's fixative for 12 minutes before storage at 4°C in 70% ethanol.

For morphological assessment, intact cellular aggregates were washed with phosphate buffered saline (PBS), fixed immediately in 4% paraformaldehyde for 30 minutes, and stored in PBS. Samples were subsequently embedded in a 2% low melting point agarose solution (30-100 µl depending on sample size) and allowed to harden at 4°C before processing, paraffin embedding, and sectioning (3 µm) on to histobond slides.

Immunostaining of single cell and paraffin-embedded sections was performed using the ABC-DAB method. Sections or cells were quenched with a 20% H_2O_2/methanol solution. Microwave antigen retrieval for cytokeratins 7 and 19, PDX-1, and Ki 67 staining involved: 15 minutes on high power (Sanyo household model, 1260W) in 800 ml Na^+ citrate for tissue sections and 5 sec. on high 6 times in 40 ml Na^+ citrate for single cells. Blocking was performed with 20% normal goat serum (Fisher) for 15 minutes. Primary antibody concentrations were as follows: 1/100 rabbit anti-human amylase (Sigma), 1/1000 guinea pig anti-porcine insulin (Dako, Denmark), 1/50 mouse anti-human CK19 (Dako), 1/200 mouse anti-human CK7 (Dako), 1/1000 rabbit anti-human PDX-1 (gift from Dr. Joel Habener, Howard Hughes Medical Institute, MA), 1/400 rabbit anti-human Mist-1 (gift from Dr. Christopher Pin, University of Western Ontario, London, Canada), and 1/50 rabbit anti-human Ki 67 (Santa Cruz Biotechnology inc., Santa Cruz, USA). Antibodies to cytokeratins 7 and 19 were used as markers for pancreatic ductal-epithelial-type cells as previously described (10, 11, 16). Primary antibody incubations were 30 minutes at room temperature followed by a wash (3X) with PBS before addition of secondary antibody. All biotinylated secondary antibodies were obtained from Vector Laboratories (Burlingame, USA) and used at a concentration of 1/200 for 20 minutes. ABC complex (Vector) incubation time was 40 minutes and visualization was with the chromagen diaminobenzadine (Biogenex, San Ramon USA) for 5 minutes. For indirect immunofluorescence of cytokeratins, primary antibodies were used at the same concentration and donkey anti-mouse Cy3 conjugated secondary antibody (Jackson ImmunoResearch Laboratories inc., Pennsylvania, USA) was used at 1/300. For detection of apoptotic cells, TUNEL staining (kit from Promega Corp., Madison, USA) was performed on dissociated cell samples from post-isolation, day 4 culture, and media change supernatant. Briefly, cells were fixed in 4% paraformaldehyde, permeabilized with 20 μg/ml proteinase K, and incubated with TdT enzyme and fluorescein-labeled dUTP for 1 hour before visualization for fluorescence. All images were captured on a Zeiss Axioskop II fluorescent microscope with a Coolsnap camera and IP lab software (Scanalytics inc., Fairfax, USA). Single cell counts were performed on a minimum of 500 cells per sample and percentages calculated as number of positive per 500. Negative controls were performed for all immunostaining procedures and consisted of the above protocol with elimination of the primary antibody (not included in figures).

For electron microscopy, aggregates and single cell suspensions were fixed in 2.5% (vol/vol) glutaraldehyde (Millonig's buffer, pH 7.2), post fixed in 1.5% (wt/vol) OsO_4, washed in distilled water, and dehydrated successively in 50, 70, 80, 90, and 100% ethanol, before embedding in aldarite. Sections were then stained with lead citrate and uranyl actetate and examined in a Hitachi H7000 (Hitachi Ltd., Tokyo, Japan) transmission electron microscope.

3. RT-PCR analysis

Samples from different donor cultures were snap-frozen dry in LN_2 and stored at –80°C for molecular analysis. Samples were re-suspended in 1 mL Trizol reagent and RNA extracted according to manufacturers protocol (Gibco). cDNA was constructed from 1 μg mRNA with 10 units (200 U/μl) Superscript reverse transcriptase in 1X buffer containing 0.01M DTT, 0.5 mM dNTPs, and 0.02 μg/μl oligo dT15. For each sample, 2 μl of cDNA was used per 25 μl reaction along with 1X PCR buffer, 2 mM MgCl, 0.2 mM dNTPs, 1 unit taq polymerase (5 U/μl), and 0.5 μM of each primer pair. All chemicals/reagents were from Gibco/Invitrogen and previously used primer sequences were obtained from Assady *et al.* (7) (PDX-1, insulin), and Heremans *et al.* (27) (neuroD, Pax4, glucagon). For all PCR reactions, 35 cycles were performed, with 30 sec. denaturation at 94°C, 30 sec. annealing at 58°C, and 30 sec. extension at 72°C. Final polymerization was at 72°C for 10 minutes followed by a 4°C hold. Products were separated on a 2% Ethidium bromide stained agarose gel and images captured on Alpha Digidoc software (Perkin-Elmer, Boston, USA). Primer sequences were as follows: 5'-CCC ATG GAT GAA GTC TAC C-3' (forward) and 5'-GTC CTC CTC CTT TTT CCA C-3' (reverse) (PDX-1-262 b.p. fragment), 5'-CCT GTA CCC CTC ATC AAG GA-3' (forward) and 5'-CTC TGT CAT CCC CAA CCA AT-3' (reverse) (Nkx6.1-182 b.p. fragment), 5'ATC CCA ACC CAC CAC CAA CC-3' (forward) and 5'-CAG CGG TGC CTG AGA AGA TT-3' (reverse) (neuroD-439 b.p. fragment), 5'-AGG AGG ACC AGG GAC TAC CGT-3' (forward) and 5'-TTT AGG TGG GGT GTC ACT CAG-3' (reverse) (Pax4-496 b.p. fragment), 5'-CCT GCA GCC CTT GGC C-3' (forward) and 5'-GTT GCA GTA GTT CTC CAG GTG-3' (reverse) (insulin-102 b.p. fragment), 5'-CCC AAG

ATT TTG TGC AGT GGT T-3' (forward) and 5'-GCG GCC AAG TTC TTC AAC AAT-3' (reverse) (glucagon-221 b.p. fragment), 5'-CTC GAG GGT AGA AAG GAT GAC GCC TC-3' (forward) and 5'-CCG AGT TGA GGT CGT GCA T-3' (reverse) (ngn3-313 b.p. fragment), 5'-TTT GTC ACC GTG GCC GTG TTT-3' (forward) and 5'-TTG CAT GTG TTC CCT GTC TGG-3' (reverse) (synaptophysin-253 b.p. fragment), 5'-CCA GCA GAG AAT GGA AAG TC-3' (forward) and 5'-GAT GCT GCT TAC ATG TCT CG-3' (reverse) (β-2 microglobulin-268 b.p. fragment). β-2 microglobulin primers were also used to verify the absence of genomic DNA contamination in samples as only the 268 b.p. fragment was seen and not the 900 b.p. genomic version of the gene. A previously characterized enriched human islet preparation was used as a positive control for several genes examined as well as for comparison with human ductal cultures. Positive controls for transiently expressed developmental factors (ie. ngn3) were samples from previous experiments found to contain the mRNA for these genes. All products were verified through sequencing using a TOPO TA cloning kit (Invitrogen) and BLAST search against known GenBank sequences (Accession numbers: NM_020999 for ngn3, NM_004048 for β-2 microglobulin, NM-000207 for insulin, NM-002054 for glucagon, NM-002500 for neuroD, NM-006168 for Nkx6.1, NM-000209 for PDX-1, NM-003179 for synaptophysin, and NM-006193 for Pax4). Negative controls consisted of cDNA from another species and Sigma water (Sigma) in place of experimental cDNA.

4. Statistical Analysis

Values are expressed as mean ± standard deviation. Statistical significance of differences was calculated by a one-way analysis of variance (ANOVA) and Scheffe's test. Linear analysis of correlations was by Pearson's correlation coefficient and all tests were performed on Statview (SAS Institute inc, Cary, NC). All significance levels were set at $p<0.05$.

II-C) RESULTS

1. Recovery of human pancreatic tissue following 4 day culture

The digested acinar fraction of the pancreas from individual donors was obtained post-islet purification and divided into different culture conditions. Dithizone staining pre-culture showed the relative absence of islets in these preparations, although no culture was completely free from islet tissue. During suspension culture extensive clumping and aggregation of tissue was observed until the second media change (day 3 culture), at which time the preparation became more dispersed. Cell survival, as measured by the change in total DNA content from immediately post-isolation to day 4 culture, was calculated from each individual donor and expressed as a mean for each culture condition (Table 2-1). When tissue was cultured in RPMI/FCS ($n = 9$), a mean survival rate of 33.1±14.2% was seen over 4 days which was higher than but not significantly different from that observed in the serum-free conditions of RPMI/ITS/BSA ($n = 12$) (30.4±12.5%) and RPMI/ITS/HSA ($n = 8$) (26.9±18.2%).

Table 2-1: Recovery of human pancreatic tissue following 4 days culture.

		% Recovery (vs. post-isolation)	
Condition	*n*	DNA	Insulin
RPMI/FCS	9	33.1±14.2[a]	8.3±8.3[b]
RPMI/ITS/BSA	12	30.4±12.5[a]	9.5±7.1[b]
RPMI/ITS/HSA	8	26.9±18.2[a]	5.0±3.6[b]

Values are mean ± S.D. of *n* different human donor preparations and are expressed as a percentage of total cellular DNA or insulin content obtained immediately after tissue isolation. [a] p<0.0001 vs. post-isolation DNA recovery; [b] p<0.0001 vs. post-isolation insulin content.

Changes in cellular insulin content as an estimation of β-cell content were also assessed immediately post-isolation to day 4 culture in each condition. The absolute amount of cellular insulin content in the acinar fraction immediately post-isolation ranged from 23 to 557 μg, which is significantly lower than the 3000 to 8000 μg obtained in purified islet preparations (unpublished observations). Following 4 day culture, the cellular insulin content was significantly lower in all culture conditions (<15%; p<0.0001) than that prior to culture (Table 2-1).

2. Cellular composition of human pancreatic tissue preparations

Cellular phenotypic changes were assessed by immunostaining for the presence of β-cells (insulin), ductal-epithelial cells (CK7/19), and acinar cells (amylase), in dissociated tissue samples on the day of isolation and after day 4 culture (Table 2-2). Immediately post-isolation, the non-purified fraction was predominately amylase-positive exocrine cells (88.6±3.1%), whereas the proportion of β-cells was only 1.8±1.4% and the percentage of CK7 and CK19 expressing cells were 20.4±21.0% and 9.9±7.7%, respectively. Culture of this tissue markedly reduced the percentage of cells staining positive for amylase in all conditions (21.3±24.0% RPMI/FCS, 16.1±18.2% RPMI/ITS/BSA, and 9.5±10.5% RPMI/ITS/HSA). In contrast, the proportion of cells expressing CK7 increased to 75.5±10.0% (RPMI/FCS), 79.5±12.6% (RPMI/ITS/BSA), and 86.7±8.5% (RPMI/ITS/HSA) while expression of CK19 increased to 59.1±15.2% (RPMI/FCS), 67.8±19.2% (RPMI/ITS/BSA), and 77.7±10.2% (RPMI/ITS/HSA). Consistent with the reduction in cellular insulin content (Table 2-1), immunostaining also revealed a decrease in the proportion of β-cells from 1.8±1.4% at day 0 to 0.5±0.6% (RPMI/FCS), 1.0±0.9% (RPMI/ITS/BSA), and 1.1±1.3% (RPMI/ITS/HSA). Moreover, the small fraction of endocrine cells in these preparations was also evidenced by the fact that in 12 different donor cultures at day 4 post-isolation, expression of the neuroendocrine marker synaptophysin was only 2.0±1.4% (not shown in table). None of the changes seen in cellular phenotype between the three culture conditions at day 4 were statistically significant.

Table 2-2: Cellular composition of human pancreatic tissue preparations immediately following isolation and after 4 days culture.

		Tissue Composition			
		β-cell	Exocrine	Ductal	
Timepoint	*n*			CK7	CK19
Post-Isolation	9	1.8±1.4	88.6±3.1	20.4±21.0	9.9±7.7
Post-Culture					
RPMI+FCS	9	0.5±0.6[a]	21.3±24.0[b]	75.5±10.0[b]	59.1±15.1[b]
RPMI+ITS/BSA	14	1.0±0.9	16.1±18.2[b]	79.5±12.6[b]	67.8±19.2[b]
RPMI+ITS/HSA	7	1.1±1.3	9.5±10.5[b]	86.7±8.5[b]	77.7±10.2[b]

Values are mean ± S.D. from *n* different human donor preparations and indicate the percentage of cells expressing either insulin (beta-cell), amylase (exocrine), or cytokeratins 7/19 (ductal). [a] $p<0.01$ vs. post-isolation; [b] $p<0.0001$ vs. post-isolation.

In addition to characterizing dissociated preparations, the cellular morphology of intact aggregates post-culture was assessed by immunostaining (Figure 2-1). It was shown that after 4 days culture, the majority of cells within the aggregates were positive for either CK7 or CK19. Amylase expression was present in some of the aggregates and very few insulin positive cells were observed.

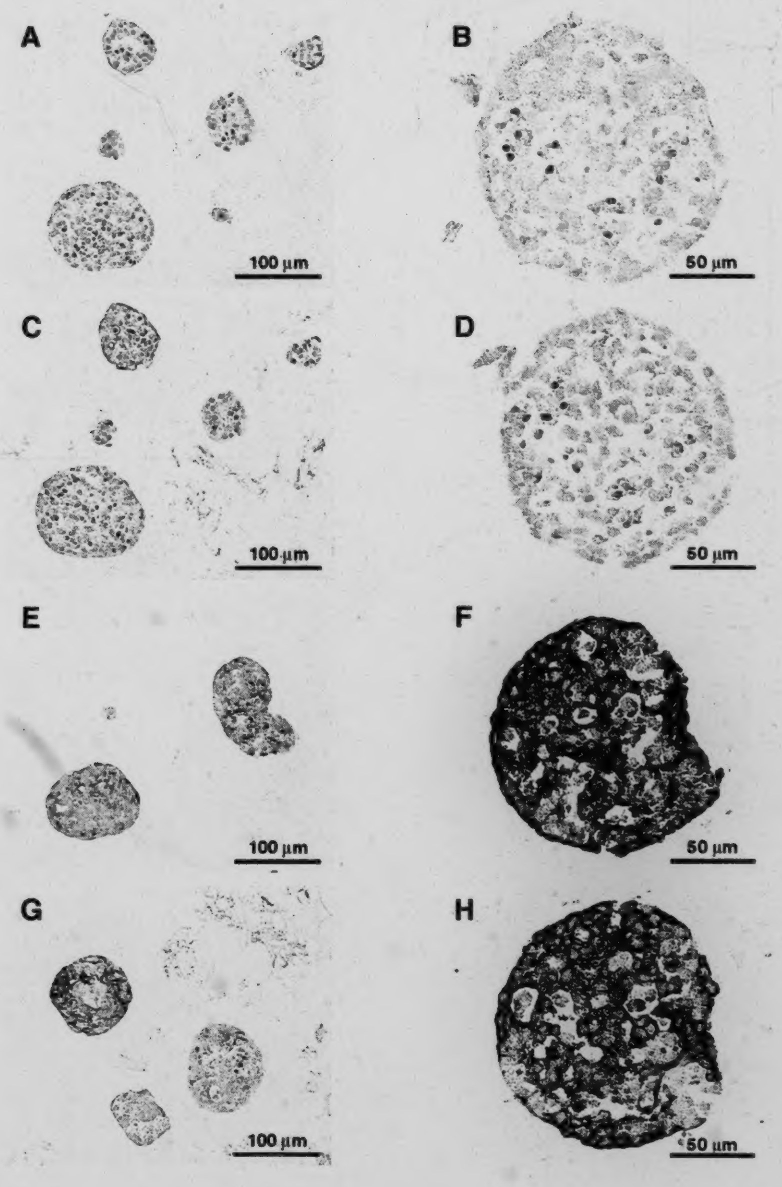
A
100 μm
B
50 μm
C
100 μm
D
50 μm
E
100 μm
F
50 μm
G
100 μm
H
50 μm

Figure 2-1: Representative immunohistochemistry for insulin **(A, B)**, amylase **(C, D)**, CK7 **(E, F)**, and CK19 **(G, H)** in human pancreatic tissue after 4 days culture in RPMI/FCS. Agarose-embedded tissue aggregates were immunostained using the immunoperoxidase method (brown) and counterstained with hematoxylin.

3. Cell Death in human pancreatic cultures

In addition to assessment for cell recovery, samples from cultures were also analyzed for evidence of cell death/apoptosis using TUNEL staining and electron microscopy. Quantification of TUNEL-positive cells in dissociated samples showed that 27.5±24.6% of the cells in culture were undergoing apoptosis at day 3-4 culture. This was significantly higher than the 4.7±3.5% ($p<0.005$) of apoptotic cells seen immediately post-isolation and represented a mean change of +30.7±26.9% over the culture period (data not shown). During media change and wash at days 1 and 3 post-isolation, tissue was centrifuged at 1200 rpm for 2 minutes. At these times, samples of wash supernatant were taken from the media supernatant after centrifugation and were found to contain a high level of apoptotic cells (~80%) indicating extensive cell death in culture. Furthermore, electron microscopy of these supernatant samples showed that a high proportion of cells were damaged, exhibiting condensed nuclei and membrane degradation (Figure 2-2).

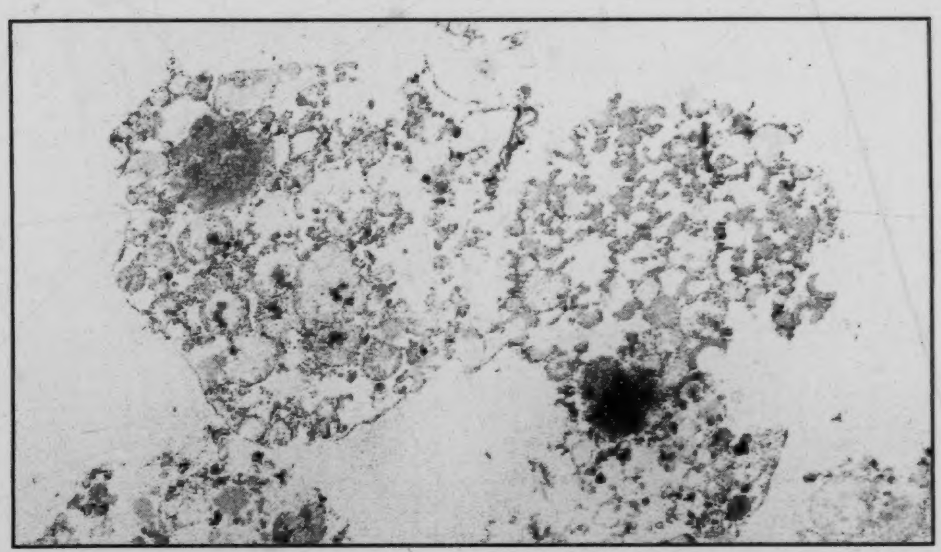

Figure 2-2: Electron microscopy of cell samples removed from the supernatant of human pancreatic cultures during media change at 2 days post-islet isolation. Magnification = 1200X.

4. Immunostaining to detect transdifferentiating acinar cells in culture

Double immunofluorescent staining was performed for CK7 and amylase at several timepoints in order to examine for possible transitional cells expressing both markers as suggestive evidence for transdifferentiation. No cells in any of the preparations (n = 9) tested were found to co-express ductal and exocrine markers. We also assessed the expression of the pancreatic acinar cell marker Mist-1 (28) in human ductal cultures in an attempt to identify possible transdifferentiating acinar cells that had lost expression of amylase and begun to express cytokeratin. Again it was found that no cells at any time-point showed co-expression of Mist-1 and either CK7 or CK19.

5. Expression of genes associated with islet neogenesis in human pancreatic ductal cultures

To assess for the possible presence of islet precursors, expression of several islet developmental-associated genes was examined by RT-PCR after 4 days culture (Figure 2-3). Cultures from 7 different donors of varying ages (lanes 1-7), as well as one previously characterized enriched human islet preparation (lane 8), were analyzed for the presence of mRNA for PDX-1, Nkx6.1, neuroD, Pax4, insulin, glucagon, neurogenin 3 (ngn3), and synaptophysin. Although little to no expression was observed of the islet developmental transcription factors ngn3 and Pax4 in the samples tested, expression of other downstream factors such as Nkx6.1 and neuroD was seen in almost all of the ductal cultures as well as in the islet preparation. Ductal samples were also found to express the genes for the mature islet proteins insulin, glucagon, and synaptophysin likely confirming the presence of a small amount of contaminating islet-endocrine tissue remaining after purification.

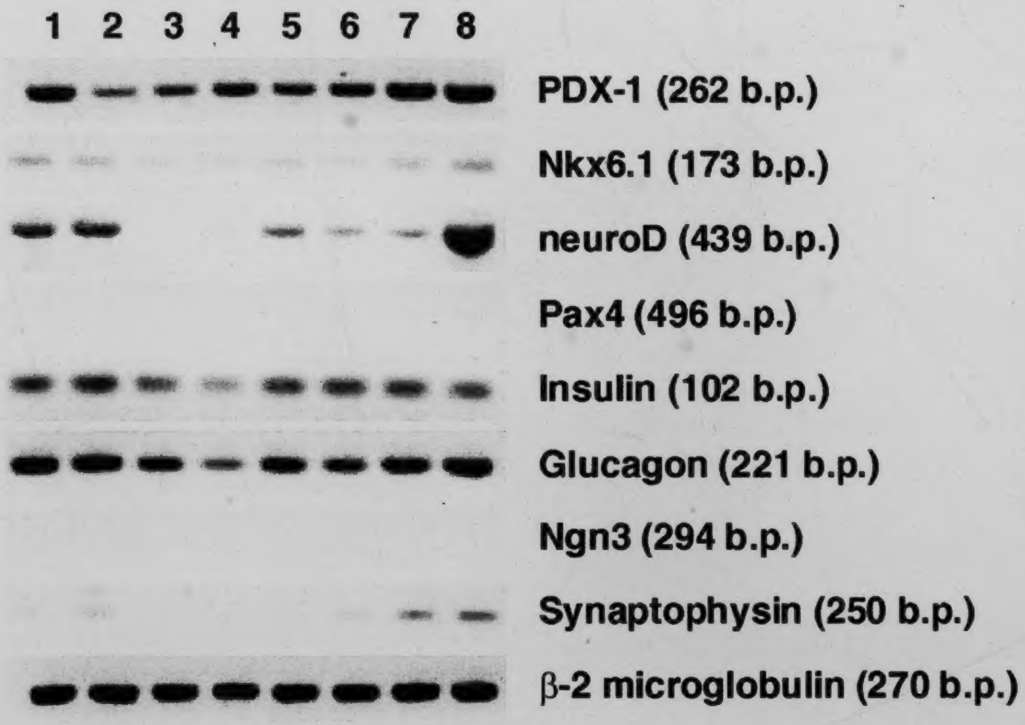
1 2 3 4 5 6 7 8
PDX-1 (262 b.p.)
Nkx6.1 (173 b.p.)
neuroD (439 b.p.)
Pax4 (496 b.p.)
Insulin (102 b.p.)
Glucagon (221 b.p.)
Ngn3 (294 b.p.)
Synaptophysin (250 b.p.)
β-2 microglobulin (270 b.p.)

Figure 2-3: Semi-quantitative RT-PCR analysis comparing samples taken from human pancreatic ductal cultures at day 4 post-isolation (lanes 1-7) and an enriched human islet preparation (lane 8). Transcription of genes such as insulin, Nkx6.1, glucagon, and synaptophysin in the majority of ductal cultures indicates the presence of contaminating islets. PDX-1 (7/7 preparations), neuroD (6/7 preparations), and Pax4 (2/7 preparations) expression indicates the possible presence of precursor cells undergoing islet differentiation, although the important developmental transcription factor ngn3 was not detected in any of the samples. β-2 microglobulin was used as a control for RNA/cDNA quality.

6. Assessment of PDX-1 expression in ductal cultures

The proportion of cells expressing the PDX-1 protein was also assessed immediately post-isolation and after 4 days culture in all culture conditions. Immunostaining and quantification in dissociated samples showed expression of PDX-1 in post-isolation fractions very similar to the proportion of β-cells (1.7±1.3% PDX-1 vs. 1.8±1.4% β-cell). Following 4 days culture, the proportion of PDX-1 positive cells varied considerably from 0.2% to 22.6%. Due to this variation, we attempted to identify a correlation with donor or isolation characteristics including age, body mass index, and organ cold ischemia time that could explain these differences amongst donors. An inverse correlation was observed between expression levels post-culture and donor age (Figure 2-4). Changes in PDX-1 expression were equivalent regardless of culture condition and the data presented is for the serum-supplemented condition. In young donors (<25 yrs.; n=7) PDX-1 expression after 4 day culture was significantly higher ($p<0.05$) than that immediately post-isolation (1.7±1.3% vs. 10.4±6.3%; range post-culture 4.6%-22.6%). Expression levels in older organs (>37 yrs.; n=6) demonstrated a lower proportion of PDX-1 (1.7%±1.3 vs. 0.9±0.5%; range post-culture 0.2%-1.4%). When data for all donor ages was combined (age range of 11-65), a direct inverse correlation was observed ($r^2 = -0.790$, $p<0.05$) with the level of PDX-1 expression in cultures at day 4. To further characterize the PDX-1 positive cells following 4 day culture, preparations were double stained for co-localization with the ductal marker CK7. In donors younger than 25 yrs, the majority of the PDX-1 positive cells were shown to also co-express CK7 (Figure 2-5 A-D). In contrast, the low numbers of PDX-1 positive cells present in the older donor cultures were shown to not co-express CK7 but co-express insulin and were thus surviving β-cells (Figure 2-5 E-H). Sections from donor pancreatic biopsies corresponding to these preparations were also assessed for PDX-1 expression and localization to assess whether the protein is normally expressed in human pancreatic ducts. As expected, PDX-1 expression in these sections was almost exclusively restricted to islets and was rarely seen in cells of ductal phenotype regardless of donor age.

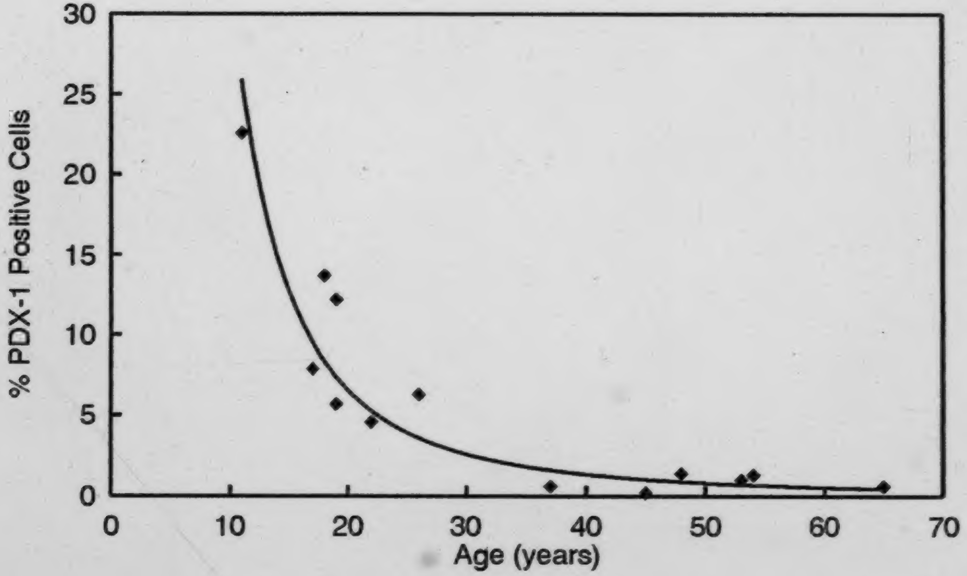

Figure 2-4: PDX-1 expression in cultured human pancreatic tissue after day 4 culture in RPMI/FCS correlates inversely with age of donor ($r^2 = -0.790$, $P<0.05$) ($n = 13$).

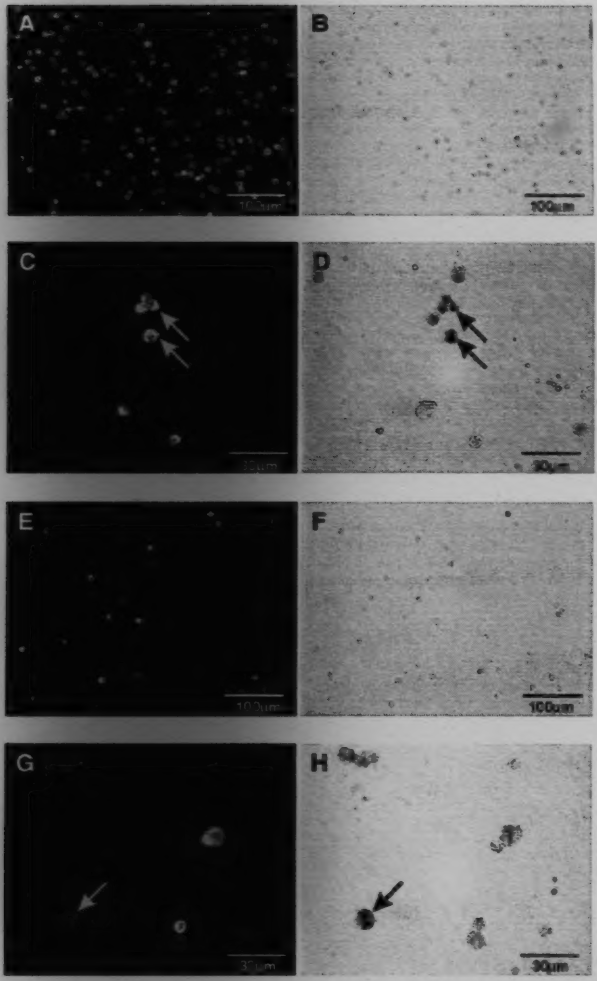

Figure 2-5: Characterization of PDX-1 expressing cells after 4 day culture of human pancreas through co-localization with insulin and the ductal marker CK7. Cytokeratin expression (bright red) and insulin expression (green) visualized with indirect immunofluorescence (**A, C, E, G**) and PDX-1 expression (brown) indicated by immunoperoxidase staining (**B, D, F, H**)). **(A-D)** Co expression of PDX-1 and CK7 in a 17 year old donor preparation post-culture indicating many double positive cells (arrows) and only a single insulin positive/PDX-1 positive cell. **(E-H)** In contrast, no co-expression of PDX-1 and CK7 in older donor preparations could be detected (arrow indicates a PDX-1 positive but CK7 negative cell) while the few PDX-1 positive cells found were also insulin positive.

7. Cell proliferation levels are low in human pancreatic ductal cultures

We also assessed the proliferative capacity of these cells at day 4 culture using immunostaining for the cell cycle marker Ki 67. Quantification revealed low numbers of replicating cells (0.6±0.7%) indicating low turnover rates for existing cells post-islet isolation.

II-D) DISCUSSION

In order for islet transplantation to become a widespread therapeutic treatment for patients with diabetes, an abundant source of islet tissue must be identified. This may be facilitated through the use of methods to produce enriched cell populations that show characteristics of ductal cells, followed by differentiation into functional endocrine cells. Transdifferentiation from an acinar to ductal phenotype has been previously proposed in both rodent and human cell culture models (11-16) although conclusive evidence to support this phenomenon has remained elusive. In the present study we have further characterized this model and derived an enriched ductal cell population following culture of human pancreatic digest. We also show evidence to favor the explanation of selective acinar cell death over transdifferentiation in these cultures and show that serum-free culture can be effective in deriving this primarily ductal population. Finally, we have shown that these cultures contain cells expressing certain markers of islet development, including PDX-1 in age-dependent manner, although the observed expression of mature-islet specific genes indicates that endocrine cells are still present and caution should be used when assessing the potential for islet neogenesis.

Due to cold ischemia of the donor pancreas followed by the stresses of enzymatic digestion, it is potentially difficult to reproducibly obtain highly viable pancreatic digest for subsequent tissue culture. This was reflected by the significant reduction in cellular DNA levels seen over 4 days culture whereby, even in the most optimal condition tested, a mean survival rate of 33.1±14.2% could be achieved. One explanation for this cell reduction is the selective loss of exocrine cells, which we have previously described in cultures of porcine pancreatic tissue (26). During the initial 24 hour culture period, many dead cells were observed which is likely the result of deleterious effects of cold storage, collagenase digestion, and Ficoll exposure. Several studies have proposed that one reason for early islet graft failure post-transplant is due to apoptosis induced during isolation (29, 30), and methods have been suggested to decrease this cell death with varying results (31, 30). Our results obtained using TUNEL staining and electron microscopy indicate that these cells do not survive well in culture, and an extensive amount of apoptosis occurs in the days following isolation. These findings are in agreement with another study using human islet cultures that showed ~32% of islet cells are apoptotic by 5 days post-isolation (29). The large variability in these experiments indicates that some donor preparations survived in culture much better than others, although a strong correlation to explain this phenomenon could not be found.

Previous reports have attributed the derivation of a ductal cell preparation from a primarily exocrine population to the phenomenon of transdifferentiation, whereby amylase positive cells de-differentiate into a phenotype resembling ductal-epithelial cells (11-16). Although this explanation seems attractive, definitive evidence for a transition of this type has remained elusive due to the difficulty in tracking cells in a heterogenous population through multiple days in culture. Another explanation for the change observed is a selective death of the exocrine cells and survival of pre-existing ductal cells resulting in a primarily ductal population after 4 days in culture. When using DNA recovery to calculate the total number of cells recovered (6.6 pg DNA/cell), and the phenotypic composition, there was an overall increase in the number of ductal cells of 170±112% (CK7) and 185±127% (CK19). Concominantly, there was a decrease in the number of amylase-positive exocrine cells to only 6.8±6.7% of the original number after 4 day culture. Considering that the overall cell population (which is predominately exocrine) is reduced by ~60% over 4 days, clearly the majority of the exocrine cell component is undergoing cell death rather than transdifferentiation. Also, because about 1% of the cells present at day 4 expressed the cell cycle marker Ki67, it is possible that the increase in number of ductal cells is at least in part due to proliferation of existing ductal cells over the total span of 4 days in culture. Thus, the final ductal cell preparation may arise primarily from survival and some proliferation of pre-existing ductal cells without ruling out the possibility of a small contribution from the transdifferentiation of a selective population of exocrine cells. However, the fact that we observed no cells at any timepoint in culture co-expressing the acinar markers amylase or Mist-1 with the ductal cytokeratin markers suggests against a direct transdifferentiation event occurring in these preparations.

The changes in phenotypic composition associated with pancreatic exocrine/ductal cell cultures have been characterized for some time (11-13). Our results agree with these previous reports in that we observe a decrease in amylase positive cells with concomitant increase in the expression of cytokeratin positive cells when culture is in serum-supplemented media. Gmyr *et al.* obtained a population containing approximately 46% CK19 and 63% CK7 expressing cells after 7 days culture and report a yield of approximately 4.9×10^7 CK7/19 positive cells per gram of tissue (12). When considering the total number of cells recovered per initial gram of digested tissue (1.81×10^8), and the percentage of CK7 positive cells, our total number of CK7 positive ductal cells recovered after 4 days culture is approximately 1.45×10^8 per gram. The difference in our higher recoveries with that of Gmyr *et al.* (12) may be because our preparations are more enriched in CK19 (70%) and CK7 (80%) positive cells. Furthermore, in the present study the culture time was 4 days whereas Gmyr *et al.* cultured for 7 days, and this longer period is most likely associated with greater cell loss.

It has been reported that the derivation of a ductal cell population from primarily exocrine cultures does not occur when serum is omitted from the culture media (14). Furthermore, all published reports utilizing this model have also utilized FCS in the media (12, 13, 16). Although, in the present study, the standard RPMI/FCS condition yielded the highest mean survival rate, this was not significantly different from the serum-free RPMI/ITS/BSA and RPMI/ITS/HSA conditions, thereby indicating that this tissue can be maintained in a serum-free environment. Moreover, our results demonstrate the successful recovery of a ductal cell population in a serum-free media. The availability of serum-free conditions will therefore allow the selection of more defined media to initiate differentiation of ductal cells into functional islets. It has also been proposed that coating of human islet grafts with xenoproteins from FCS in pre-transplant media may accelerate alloimmune destruction (1), and for this reason serum-free culture is desirable for cells which may eventually be used in a clinical application.

It has been proposed that pancreatic ductal cells are islet progenitors and that these cultures can be used to create an abundant supply of new islets for transplantation (9, 10, 27). Recently, Heremans *et al.* showed the differentiation of a proportion (~12%) of these cells to insulin-producing cells using viral transduction of the transcription factor ngn3 (27). These types of studies, however, are based on the assumption that pre-existing islets have been removed during isolation and observed neogenesis is not due either to β-cell replication or de-differentiation and subsequent re-differentiation of mature islet cells. We have shown, however, using immunostaining and RT-PCR, that islet-endocrine cells are still present in these cultures. Most notably, the presence of the hormones insulin and glucagon indicates that differentiated islet cells exist and must be considered when assessing subsequent neogenesis. For this reason, care should be taken to use only the donor preparations most free of contaminating islets as starting material to induce ductal to islet differentiation. The presence, however, of transcription factors known to be expressed transiently in islet development and not in mature islet cells (e.g. neuroD), combined with the observed re-expression of PDX-1 in a select population of ductal cells suggests that the potential for islet neogenesis from true precursor cells does exist in these cultures.

The donor age dependent re-expression of PDX-1 in culture by ductal cells is an important finding when considering plasticity in the adult pancreas. PDX-1 is expressed in all pancreas-dedicated cells during embryonic development regardless of future phenotype (32) and is maintained primarily in β-cells of the mature organ (33). PDX-1 is necessary for proper pancreatic formation as mice that are homozygous for the null mutation are born without a pancreas and die within a few days (19). Using semi-quantitative PCR techniques, Gmyr *et al.* report a 10.5-fold increase in PDX-1 mRNA levels in human pancreatic cultures after 2 days with a 3.2-fold increase in protein expression (16) while Bouwens *et al.* describe a similar up-regulation in rat tissue (14). Our results using quantitative immunohistochemistry to detect PDX-1 positive cells show an increase following 4 day culture in both conditions but only in certain donor preparations. We therefore examined various donor characteristics and found that the level of increase of PDX-1 in these cultures is dependent on donor age. This is an important finding to take into account when assessing the potential of future donor organs for islet neogenesis. In particular, all organs from donors under 25 yrs showed an increased proportion of PDX-1 positive cells post-culture while those from older donors maintained or demonstrated decreased levels of expression. We also confirmed, as reported previously (16), that the cells expressing PDX-1 are ductal and co-express CK7/19. We also, however, demonstrate that PDX-1/CK7 co-expression is present only in tissue prepared from donors <25 yrs, since in older donors this co-localization was not observed. The increase in PDX-1 expression seen in this study may indicate a higher degree of plasticity in younger organs. These findings are of particular interest in light of a recent study by Pipeleers and coworkers (34), which suggests that human ductal cultures derived from young donors (<10 years) exhibit a greater potential for islet neogenesis when transplanted in a nude mouse model. While the authors of this study do not provide a clear mechanism by which younger cells undergo increased levels of differentiation, it can be proposed from our results that a greater proportion of the ductal population from young pancreases are capable of expressing PDX-1 under the appropriate conditions, and thus initiating the pathway to islet cell phenotype.

Due to conflicting reports regarding the levels and ductal localization of PDX-1 in the adult pancreas (35, 36), we assessed pancreatic biopsies from donors of all cultures represented in this study for PDX-1 expression. Immunostaining of corresponding biopsies collected pre-islet isolation showed that PDX-1 is rarely expressed in ductal cells in vivo regardless of donor age, reinforcing the proposal that young donor tissue may have greater plasticity under abnormal conditions (ie. culture). These findings are significant because a recent report by Gu *et al.* (37) suggests that ductal progenitor cells, which transienty express PDX-1 during embryogenesis then turn off PDX-1 and become ductal-epithelial cells, may not necessarily be endocrine/exocrine precursors in the developing pancreas. We propose that mature ductal cells which are able to re-express PDX-1 may be capable of reverting back to stages of embryonic development and may in fact be able to change lineage and act as islet precursors. Further studies are being undertaken to assess the fate and plasticity of these PDX-1 expressing ductal cells under different conditions.

This conclusion that ductal cells act as islet precursors is not in agreement with a recent paper by Melton and co-workers (38), in which they proposed that β-cell neogenesis in the adult pancreas is due to the replication of pre-existing ß-cells and not from the differentiation of a precursor. Our data is not in disagreement with the Melton results for two reasons. Firstly, we clearly demonstrate that PDX-1 re-expression in the ductal cells is age dependent. Therefore it is possible that, in the young pancreas, ductal cells have a greater potential for differentiation into ß cells that is reduced or absent in adult stages. Secondly, we show the presence of islet β-cells even in cultures derived from the most dense gradient layer (exocrine) after islet purification. This suggests that in all studies to date reporting ductal cell differentiation, the possibility of only β-cells giving rise to new β-cells cannot be ruled out. However, although the results from Melton and colleagues are compelling, there remains convincing evidence from other groups to suggest that ductal cells are capable of differentiating into ß-cells (9, 10, 27, 34). At this time, it is reasonable to assume from all available evidence that β-cell turnover in the adult pancreas may occur in part from existing β-cell replication and in part from unipotent or multipotent ductal cell precursors.

In summary, we have characterized the transition from a primarily exocrine cell pancreatic culture to a highly enriched ductal-epithelial cell preparation. The novel ability to culture this tissue in a serum-free environment allows the consideration of possible future clinical applications involving this tissue. In addition, we have shown that ductal-like cell populations prepared from younger donors contain a higher proportion of PDX-1 positive cells and propose that this tissue may have a greater potential to differentiate into functional islet endocrine cells. However, problems such as extensive cell death may hamper these efforts and it remains to be demonstrated whether this tissue can be converted to an abundant supply of functional islets for transplantation.

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CHAPTER III

ADULT HUMAN PANCREATIC DUCTAL CELLS INDUCED TO EXPRESS NEUROGENIN 3 PARTIALLY DIFFERENTIATE *IN VITRO* AND FULLY DIFFERENTIATE *IN VIVO* TO INSULIN-PRODUCING PHENOTYPE

III-A) INTRODUCTION

Islet transplantation is now a clinical alternative for certain patients with type 1 diabetes (1-3). However, for this treatment to become widely applicable an abundant source of islet tissue must be identified in order to treat the growing number of diabetic patients. Several alternatives have been proposed for this source (4-6), one of the most promising being the induction of adult pancreatic stem cell populations to islet cell differentiation. Although a putative islet progenitor cell has yet to be identified, strong evidence exists to suggest that pancreatic ductal-epithelial cells play a role in islet neogenesis and may be capable of differentiation to islet-endocrine phenotype (7, 8). Furthermore, this lab (9) and others (10-13) have shown that a relatively pure population of ductal cells can be obtained through culture of the non-endocrine component of the pancreas after collagenase digestion. Despite this evidence, to date a clinically relevant amount of insulin-producing tissue has not been produced from pancreatic ductal cells.

Several groups have used viral gene transduction in an attempt to induce pancreatic or other cell types to β-cell differentiation. Transduction of the insulin gene itself has been shown in gut K-cells (14), as well as in primary muscle cells and cell lines (15), however problems involving reliable gene delivery and sustained expression limit the usefulness of this approach. A more effective way to use gene delivery to drive islet neogenesis may be to induce the expression of transient developmental transcription factors to turn on the islet differentiation pathway and induce permanent cell differentiation. For example, induction of the transcription factor PDX-1 in liver cells has been shown to drive these cells to insulin production (16, 17). Similarly, a recent study by Heremans *et al.* (18) showed that ectopic expression of the transcription factor neurogenin 3 (ngn3) in human pancreatic ductal cells induced differentiation to β-cell phenotype; characterized by the induction of several downstream islet developmental transcription factors, increased insulin content, and higher proportion of insulin-positive cells in these cultures. Although in this study only roughly 13% of the ductal cells were converted to β-cell phenotype, these findings suggest that inducing expression of ngn3 is an effective technique to drive differentiation from pancreatic ductal cells.

Ngn3 is a basic helix-loop-helix (bHLH) transcription factor involved in CNS and pancreatic cell differentiation (19, 20). The gene acts to regulate expression of other downstream transcription factors leading to expression of insulin and other β-cell specific genes (21, 22). Furthermore, it has been reported that ngn3 expressing cells are islet progenitors during pancreatic development (23, 24), and that the absence of the gene results in no formation of the endocrine component of the pancreas (25). The aim of this study was to induce expression of ngn3 in human pancreatic ductal cultures using viral-mediated gene delivery and to confirm the resulting β-cell differentiation reported by Heremans *et al.* (18). In addition, it was examined whether *in vivo* conditions could provide a more suitable environment for islet-endocrine differentiation from human ductal cells, and whether these differentiated cells could provide control of diabetes in an animal model. The hypothesis of the study was that a greater level of islet cell differentiation can be achieved through transplant of undifferentiated, ngn3-expressing human pancreatic ductal cells as compared to that achieved in previous studies utilizing *in vitro* conditions for differentiation (18).

III-B) MATERIALS AND METHODS

1. Tissue procurement and culture

All experimental protocols used in this study were approved by the Research Ethics Board of the University of Alberta. Human donor pancreases were removed from cadaveric donors with informed research consent from relatives and processed according to the protocols described by this laboratory (1, 26). Briefly, organs were cold stored in University of Wisconsin solution and perfused via the duct with the enzyme solution Liberase (Roche, Indianapolis, USA). Once digested, islets were purified on continuous Ficoll gradients using the refrigerated Cobe 2991 (COBE BCT inc., Lakewood, USA) blood cell processor. Immediately following islet purification, the predominately acinar cell fraction was collected from the Cobe bag and washed three times with Hanks balanced salt solution (HBSS; Sigma-Aldrich, Oakville, Canada) supplemented with 0.5% bovine serum albumin (BSA; fraction V, Sigma). After dithizone staining of representative samples to assess islet content, tissue was cultured in suspension with approximately 500 µl "pack tissue volume" per non-treated 15 cm plate (Fisher Scientific, Edmonton, Canada) in RPMI 1640 medium (Gibco/Invitrogen, Burlington, Canada) supplemented with 1% insulin-transferrin-selenium (ITS) and 0.5% human serum albumin (HSA) (all supplements/sera from Sigma). Media changes were performed at day 1 and day 3 post-culture. This serum-free protocol has been previously confirmed to result in an enriched population of ductal-epithelial cells after 4 day culture (9) that were subsequently used for viral transduction experiments. Islets from Balb/c mice for co-transplant were isolated according to a modification of the technique originally described by Lacy and Kostianovsky (27). Briefly, pancreases were distended via cannulation of the common bile duct with a 2 mg/ml filter-sterilized collagenase (Sigma)/HBSS solution and removed for digestion. After trimming of the spleen and other material from the pancreas, collagenase digestion was carried out for ~12 minutes in a 37°C water bath before aspiration, filtration, and separation of islets using a discontinuous dextran gradient. Mouse islets were cultured overnight before transplant in all experiments.

2. Adenoviral transduction of human ductal cells

Human ductal cultures were transduced with a replication-deficient adenovirus containing a vector with the genes for enhanced green fluorescent protein (eGFP) and murine ngn3 driven by separate CMV promoters (gift from Dr. Harry Heimberg, Free University of Brussels, Belgium) using a modification of a previously described protocol (18). Briefly, ductal aggregates were pre-treated in Ca^{2+}-free media supplemented with 1 mM EGTA and 0.5% BSA with frequent pipetting for 10 minutes before infection to break down extracellular matrix and allow access of virus particles to cells within the aggregates. Preliminary experiments showed this treatment to significantly increase infection efficiency over untreated aggregates (data not shown). Next, cells were seeded at 10 x 10^6 per plate in 5 ml of Hams-F10 media supplemented as previously described (4). These cultures were infected at a multiplicity of infection (MOI) of 50 with the adeno-ngn3 virus for 4 hours at 37ºC, and were subsequently washed two times with media to remove virus particles and re-cultured in Hams-F10 for 24 hours. Preliminary infection efficiency was judged by visualization of GFP expression on a Zeiss Axiovert 25 inverted fluorescent microscope (Carl Zeiss Canada, ON, Canada) with a Coolsnap camera and IP lab software (Scanalytics inc., Fairfax, USA).

3. *In vitro* assessment of transduced cultures

After isolation, 4 day tissue culture, and at selected timepoints after viral transduction, preparations were assessed for cellular insulin and DNA content as well as cell composition according to methods previously described [9, 13]. To determine cellular composition, aggregates were dissociated into single cell suspensions to facilitate quantification of immunostaining. Single cell suspensions were obtained by mechanical disruption using pipetting at 37°C in Ca^{2+}-free media supplemented with 1 mM EGTA and 0.5% BSA for 7 minutes before addition of trypsin (25 µg/ml) and DNAse (4 µg/ml) and further pipetting for 4 minutes. Cells were then allowed to adhere to histobond slides (Marienfeld, Germany) and fixed in Bouin's fixative for 12 minutes before storage at 4°C in 70% ethanol.

Immunostaining of single cell preparations and removed grafts was performed using the ABC-DAB method (Avidin-Biotin complex visualized with the chromagen diaminobenzidine). Sections or cells were quenched with a 20% H_2O_2/80% methanol solution. Microwave antigen retrieval for ngn3, cytokeratin 7 (CK7), Ki67, and synaptophysin staining involved: 15 minutes on high power (Sanyo household model, 1260W) in 800 ml Na^+ citrate for tissue sections and 5 seconds on high 6 times in 40 ml Na^+ citrate for single cells. Blocking was performed with 20% normal goat serum

(Fisher) for 15 minutes. Primary antibody concentrations were as follows: 1/100 rabbit anti-human amylase (Sigma), 1/1000 guinea pig anti-porcine insulin (Dako Diagnostics Canada inc., Mississauga, Canada), 1/50 mouse anti-human CK19 (Dako), 1/200 mouse anti-human CK7 (Dako), 1/50 rabbit anti-human synaptophsin (Dako), 1/50 rabbit anti-human Ki 67 (Santa Cruz Biotechnology inc., Santa Cruz, USA), 1/50 rabbit anti-human c-peptide (Linco Research inc., St. Charles, MI), and 1/1000 rabbit anti-mouse ngn3 (gift from Dr. Michael German, University of California, CA). Primary antibody incubations were 30 minutes at room temperature followed by wash (3X) with PBS before addition of secondary antibody. All biotinylated secondary antibodies were obtained from Vector Laboratories (Burlingame, USA) and used at a concentration of 1/200 for 20 minutes. ABC complex (Vector) incubation time was 40 minutes and visualization was with the chromagen diaminobenzidine (Biogenex, San Ramon USA) for 5 minutes. Indirect double immunofluorescence was performed sequentially with primary antibodies used at the same concentration. Donkey anti-mouse Cy3, goat anti-mouse FITC, goat anti-rabbit Cy3, and goat anti-guinea pig FITC conjugated secondary antibodies (all from Jackson ImmunoResearch Laboratories inc., Pennsylvania, USA) were used at 1/200. All images were captured on a Zeiss Axioskop II fluorescent microscope (Carl Zeiss Canada) with a Coolsnap camera and IP lab software (Scanalytics inc.). Single cell counts were performed on a minimum of 500 cells per sample and percentages calculated as number of positive per 500. Negative controls were performed for all immunostaining procedures and consisted of the above protocol with elimination of the primary antibody (not included in figures).

4. RT-PCR analysis

Samples from different donor cultures were suspended in trizol reagent and stored at –80°C for molecular analysis. Samples of whole grafts removed from transplanted animals were pulverized in Eppendorf tubes using microcentrifuge pestles (Rose Scientific, Edmonton, Canada) before addition of Trizol and freezing. All samples were subsequently thawed and RNA extracted according to the *manufacturers* protocol (Gibco). cDNA was constructed from 1 μg mRNA with 10 units (200 U/μl) Superscript reverse transcriptase in 1X buffer containing 0.01M DTT, 0.5 mM dNTPs, and 0.02 μg/μl oligo dT15. For each sample, 2 μl of cDNA was used per 25 μl reaction along with 1X PCR buffer, 2 mM MgCl, 0.2 mM dNTPs, 1 unit taq polymerase (5 U/μl), and 0.5 μM of each primer pair. All chemicals/reagents were from Gibco/Invitrogen and previously used primer sequences were obtained from Assady *et al.* (28) (PDX-1, insulin), and Heremans *et al.* (18) (neuroD, Pax4, glucagon). For all PCR reactions, 35 cycles were performed, with 30 sec. denaturation at 94°C, 30 sec. annealing at 58°C, and 30 sec. extension at 72°C. Final polymerization was at 72°C for 10 minutes followed by a 4°C hold. Products were separated on a 2% ethidium bromide stained agarose gel and images captured with Alpha Digidoc software (Perkin-Elmer, Boston, USA). Primer sequences were as follows: 5'-CCC ATG GAT GAA GTC TAC C-3' (forward) and 5'-GTC CTC CTC CTT TTT CCA C-3' (reverse) (PDX-1-262 b.p. fragment), 5'-CCT GTA CCC CTC ATC AAG GA-3' (forward) and 5'-CTC TGT CAT CCC CAA CCA AT-3' (reverse) (Nkx6.1-182 b.p. fragment), 5'ATC CCA ACC CAC CAC CAA CC-3' (forward) and 5'-CAG CGG TGC CTG AGA AGA TT-3' (reverse) (neuroD-439 b.p. fragment), 5'-AGG AGG ACC AGG GAC TAC CGT-3' (forward) and 5'-TTT AGG

TGG GGT GTC ACT CAG-3' (reverse) (Pax4-496 b.p. fragment), 5'-CCT GCA GCC CTT GGC C-3' (forward) and 5'-GTT GCA GTA GTT CTC CAG GTG-3' (reverse) (human insulin-102 b.p. fragment), 5'-CCC AAG ATT TTG TGC AGT GGT T-3' (forward) and 5'-GCG GCC AAG TTC TTC AAC AAT-3' (reverse) (glucagon-221 b.p. fragment), 5'-CTC GAG GGT AGA AAG GAT GAC GCC TC-3' (forward) and 5'-CCG AGT TGA GGT CGT GCA T-3' (reverse) (human ngn3-313 b.p. fragment), 5'-TTT GTC ACC GTG GCC GTG TTT-3' (forward) and 5'-TTG CAT GTG TTC CCT GTC TGG-3' (reverse) (synaptophysin-253 b.p. fragment), 5'-CCA GCA GAG AAT GGA AAG TC-3' (forward) and 5'-GAT GCT GCT TAC ATG TCT CG-3' (reverse) (human β-2 microglobulin-268 b.p. fragment), 5'- -3' (forward) and 5'- -3' (reverse) (mouse/human GAPDH- b.p. fragment). β-2 microglobulin primers were also used to verify the absence of genomic DNA contamination in samples as only the 268 b.p. fragment was seen and not the 900 b.p. genomic version of the gene. A previously characterized enriched human islet preparation was used as a positive control for several genes examined as well as for comparison with human ductal cultures. Positive controls for transiently expressed developmental factors were samples from previous experiments found to contain the mRNA for these genes. All products were verified through sequencing using a TOPO TA cloning kit (Invitrogen) and BLAST search against known GenBank sequences (Accession numbers: NM_020999 for ngn3, NM_004048 for β-2 microglobulin, NM-000207 for insulin, NM-002054 for glucagon, NM-002500 for neuroD, NM-006168 for Nkx6.1, NM-000209 for PDX-1, NM-003179 for synaptophysin, and NM-006193 for Pax4). Negative controls consisted of both cDNA absent for the gene of interest and Sigma water (Sigma) in place of experimental cDNA.

5. Animals and transplantation experiments

Infected/control human ductal tissue and isolated Balb/c mouse islets were transplanted to immunocompromised C57BL/6-rag1$^{-/-}$ knockout mice (purchased from Dr. Colin Anderson, University of Alberta, Canada) for *in vivo* differentiation experiments. Animals were rendered diabetic two days before transplant using streptozotocin (Sigma) administered intraperitoneally (i.p.) at 175 mg/kg and were confirmed to be hyperglycemic (blood glucose >20 mmol/L) by pre-transplant monitoring (OneTouch Ultra^{TM} blood glucose monitors, Lifescan inc., Milpitas, CA). Mice were anesthetized using halothane and all grafts were transplanted in a single operation. Balb/c islet grafts (approximately 250 islets) were transplanted under the left renal capsule using a micromanipulator syringe as previously described (4). Subsequently, the pancreas was reached via a separate vertical midline incision and the human graft (5-10 x 10^6 cells) was delivered into the body of the pancreas near the stomach using a micromanipulator syringe through a small incision followed by cauterization to ensure minimal leakage from the diffuse organ. In another series of experiments, some mice received infected/control human tissue under the right renal capsule while still receiving mouse islets under the left renal capsule. All transplanted mice were monitored for blood glucose daily for 2-3 days post-transplant and weekly thereafter.

At 20 day intervals post-transplant, one animal from each experimental group underwent a survival nephrectomy to remove the kidney containing the Balb/c islet graft. Subsequently, these mice were monitored for blood glucose for 3-5 days before sacrifice and removal of the human tissue graft for assessment. Grafts were located in the mouse pancreas by examining the area of transplant for the cauterization scar, and subsequently locating the human tissue, which was yellow in colour as compared to the surrounding mouse tissue. Grafts were trimmed, and either placed in Z-fix for >24 hrs., embedded in paraffin, and sectioned at 3 μm thickness on to histobond slides, or suspended in trizol reagent as described in the "RT-PCR analysis" section for subsequent molecular analysis. Alternatively, when visualization of GFP expression in grafts was necessary grafts were placed in Cryomatrix medium (ThermoShandon, Pittsburgh, PA), frozen at –80 °C, and cryosectioned on to histobond slides. Immunostaining of grafts was done using the ABC-DAB method as described above.

6. Statistical Analysis

All values are reported as mean ± S.D. Statistical comparison of phenotypic proportions between in vitro control and infected grafts at different timepoints was done using ANOVA with post-hoc Scheffes test.

III-C) RESULTS

1. Transduction and *in vitro* assessment of human pancreatic ductal tissue.

Enriched human ductal preparations used for infection (n = 11) were derived through culture of non-endocrine pancreatic digest as described by this lab (9) and others (12, 13). These cultures consisted of 86.7±8.5% ductal (CK7 positive), 9.5±10.5% exocrine (amylase positive), and <1% β-cells (insulin-positive). Observed under phase-contrast microscopy, cellular aggregates were surrounded by well-defined capsules and showed little evidence of necrosis. After treatment with dissociation media for 10 minutes, aggregates appeared smaller and more fragmented, allowing for better access of viral particles and increased infection efficiency (confirmed in preliminary experiments). After adenoviral transduction (24 hrs), expression of EGFP was observed extensively throughout nearly 100% of infected cellular aggregates (Figure 3-1). However, subsequent immunostaining of dissociated cell samples from 24 hours post-infection showed that only 14.0±8.8% of transduced cells expressed the murine ngn3 transgene (Figure 3-2).

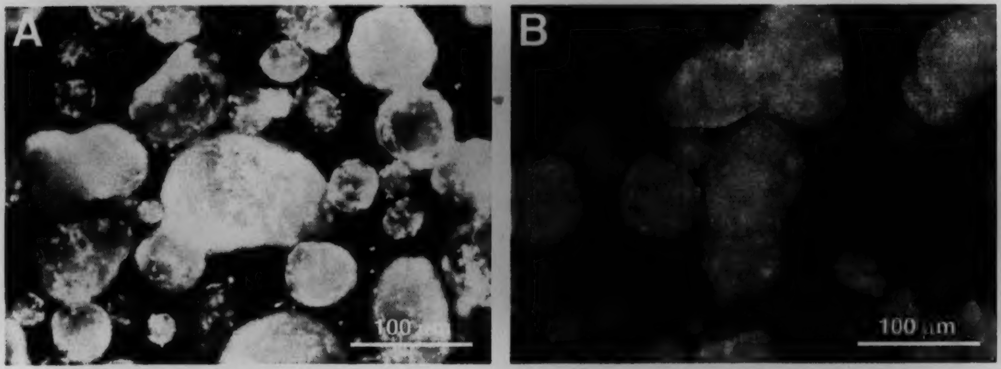


Figure 3-1: EGFP expression in transduced (A) and control (B) human pancreatic ductal aggregates at 24 hours post-infection.

Figure 3-2 shows the phenotypic composition of infected and control cultures at pre-infection, 48 hours post-infection, and 10 days post-infection. The majority of cells (80-90%) remained ductal in phenotype (CK19 positive) at both 48 hours and 10 days post-infection (not shown). In contrast to results reported by Heremans *et al.* (18), the percentage of insulin-positive cells did not increase over the culture period, remaining at <1% in both infected and control cultures. There was, however, a significant increase in the proportion of cells expressing the neuroendocrine marker synaptophysin from

0.5±0.2% pre-infection to 2.5±1.0 at 48 hours and 5.5±2.9% at 10 days post-infection ($p<0.005$). This was significantly higher than control cultures at 10 days (2.0±0.9%; $p<0.05$). Due to extensive cell death beyond 10 days culture, assessment of differentiation was not continued further *in vitro*.

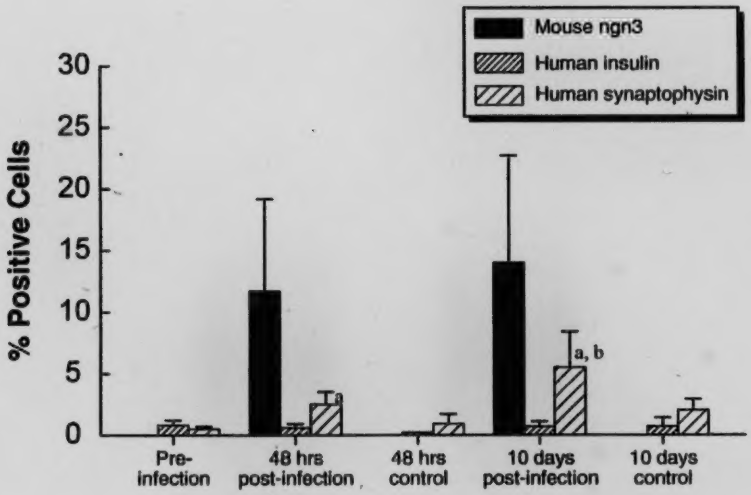
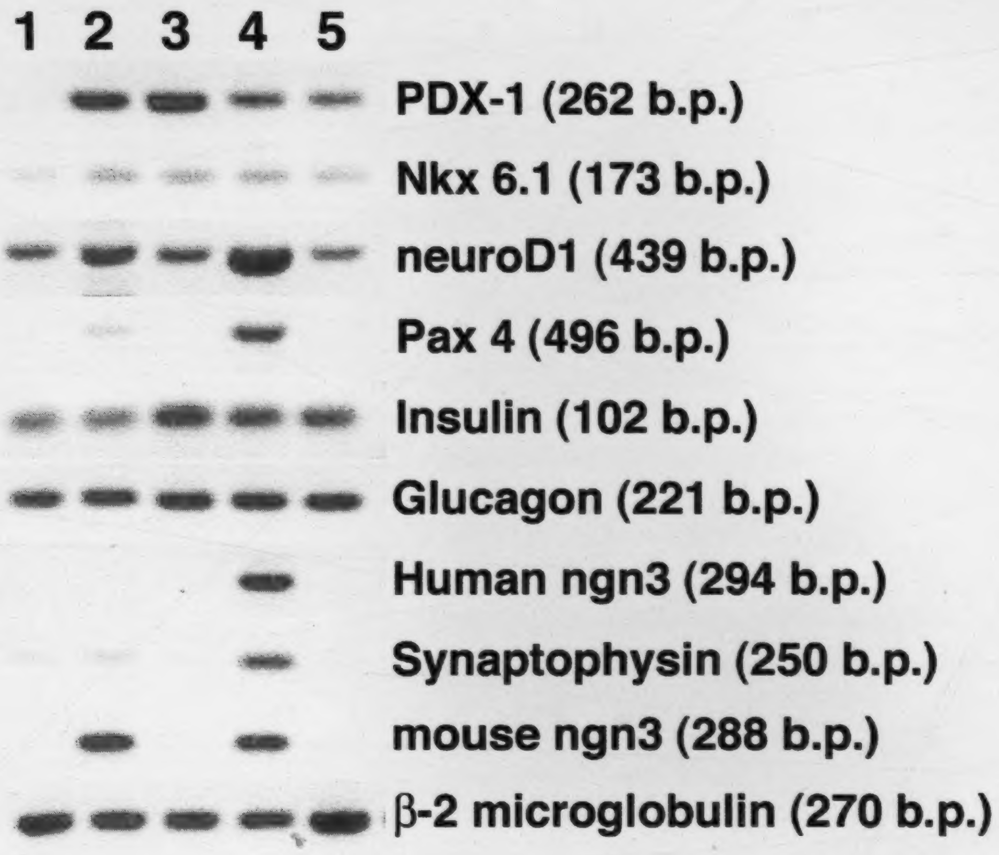


Figure 3-2: Phenotypic composition of control and transduced human pancreatic cultures *in vitro* at pre-infection, 48 hours post-infection, and 10 days post-infection. Cultures were assessed for expression of insulin (β-cells), synaptophysin (neuroendocrine marker), and murine ngn3 (transgene). Values are expressed as the mean percentage of positive cells ± S.D. [a] $p<0.005$ vs. synaptophysin expression pre-infection. [b] $p<0.05$ vs. synaptophysin expression in 10 day control cultures.

RT-PCR was performed on samples prior to infection, post-infection (48 hours and 10 days), and control cultures to detect the transcription of developmental and islet-associated genes (Figure 3-3). Importantly, it is evident that although these cultures contain <1% islet endocrine cells by immunostaining, this is sufficient to give a positive result by PCR for insulin and glucagon at all timepoints tested in all preparations used (Figure 3-3). This analysis also confirmed the expression of the murine-specific ngn3 transgene in infected, but not pre-infection or control cultures. mRNA for the transcription factor PDX-1 was detected sporadically before infection and was usually present subsequently in both control and infected cultures, indicating that PDX-1 induction was not a specific effect of exogenous ngn3 expression. However, induction of Pax4 was seen only in infected cultures and not in corresponding controls, and similarly an up-regulation of neuroD was observed in infected as compared to control cultures. In addition, expression of human ngn3 was detected weakly at 48 hours, and strongly at 10 days in infected but not control cultures. Preliminary experiments as well as these results confirmed the specificity of human and murine ngn3 primers used in these experiments. RNA/cDNA integrity was confirmed in all samples tested using the ubiquitously expressed human β-2 microglobulin gene.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PDX-1 (262 b.p.)
Nkx 6.1 (173 b.p.)
neuroD1 (439 b.p.)
Pax 4 (496 b.p.)
Insulin (102 b.p.)
Glucagon (221 b.p.)
Human ngn3 (294 b.p.)
Synaptophysin (250 b.p.)
mouse ngn3 (288 b.p.)
β-2 microglobulin (270 b.p.)

Figure 3-3: RT-PCR analysis of human pancreatic samples from transduced and control *in vitro* cultures. Lane 1: pre-infection, lane 2: 48 hours post-infection, lane 3: 48 hours non-infected control, lane 4: 10 days post-infection, lane 5: 10 day non-infected control. Samples were assessed for transcription of human PDX-1, Nkx6.1, neuroD, Pax4, insulin, glucagon, ngn3, and synaptophysin, as well as murine specific ngn3. RNA/cDNA integrity was confirmed by detection of the housekeeping gene β-2 microglobulin.

2. *In vivo* differentiation of ngn3-expressing human ductal tissue.

In order to assess the potential for *in vivo* differentiation, transduced cells were transplanted into diabetic immune deficient C57BL/6-rag1$^{-/-}$ mice. Mean blood glucose level at the time of transplant was 29.7±0.8 mmol/L. In order to maintain survival and control hyperglycemia in diabetic animals, approximately 250 Balb/c islets were implanted under the left renal capsule at the time of human ductal transplant. In all cases, excluding incidences of technical error, this resulted in a return to normoglycemia (<10 mmol/L) and prolonged survival, yet provided a model of pancreatic damage to test ductal graft differentiation.

Animals received 5-10 x 10^6 transduced human ductal cells directly into the pancreatic parenchyma along with the renal Balb/c islet graft described above. To confirm engraftment and short-term survival, some grafts were removed 7 days post-transplant (ie. 10 days post-infection) for histological analysis. Similar to 10 day *in vitro* cultures, the grafts showed no evidence of differentiation into insulin-positive cells, remained strongly positive for CK19, and continued to express eGFP as well as the murine ngn3 transgene (Figure 3-4). These results demonstrate that transduced human ductal tissue can successfully engraft in the mouse pancreas with continued transgene expression.

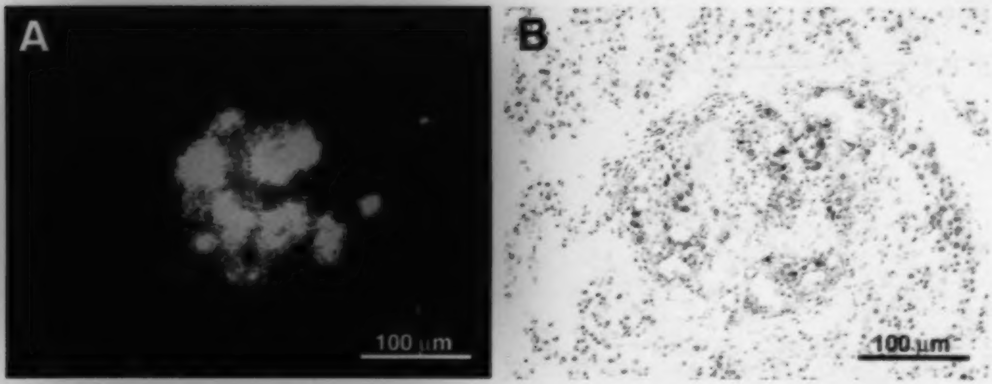

Figure 3-4: Immunostaining of intra-pancreatic transduced human grafts removed at day 7 post-transplant (day 10 post-infection). Expression of eGFP (**A**) and murine ngn3 (**B**) persist *in vivo* after engraftment.

In long term transplants (up to 100 days), animals initially returned to normoglycemia within 1-3 days (due to Balb/c graft) with one animal dying before 20 days post-transplant. At 20 day intervals, one mouse from each condition underwent removal of the Balb/c islet graft (survival nephrectomy) to determine whether normoglycemia would persist due to differentiation of the human ductal cells. However, in all mice removal of the Balb/c islets resulted in a return to hyperglycemia within 2-4 days (Figure 3-5). Animals were subsequently sacrificed 3-5 days after nephrectomy and the human graft was identified, dissected from surrounding murine pancreatic tissue, and fixed for histological analysis. Interestingly, in most cases beyond 20 days post-transplant, grafts were found to be well vascularized when observed under a 30X dissecting microscope (Figure 3-6).

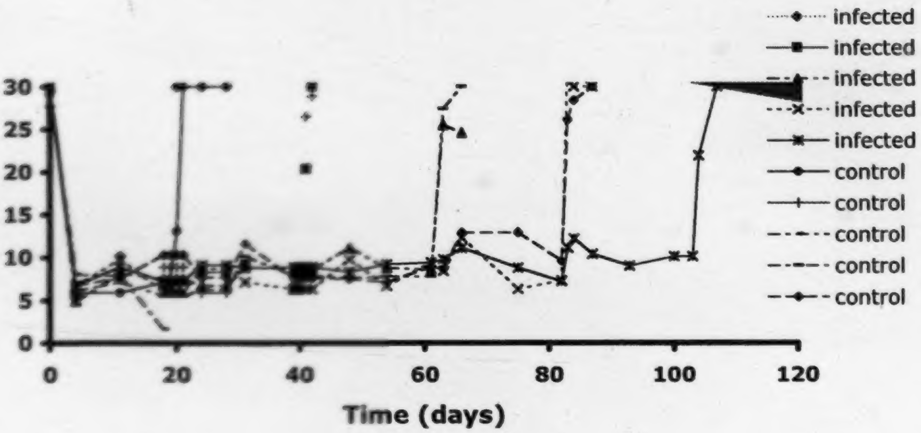

Figure 3-5: Blood glucose levels (mmol/L) in 10 mice transplanted with either ngn3-infected (n = 5) or non-infected control (n = 5) human pancreatic ductal tissue over 100 days post-transplant. One animal from each condition underwent survival nephrectomy followed 3-5 days later by termination and graft removal at 20 day intervals.

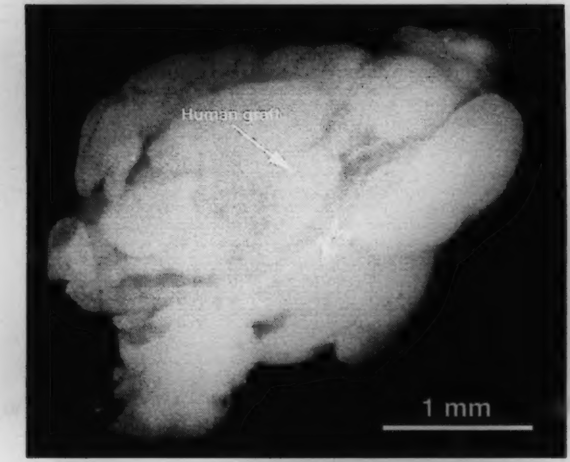

Figure 3-6: Vascularization of transduced intra-pancreatic human ductal graft removed at day 15 post-transplant. Human graft (arrow) is identified by a yellowish color as opposed to lighter colored murine pancreatic tissue.

Human ductal grafts from infected and control animals were assessed using immunostaining. Although a large proportion of the tissue present in stained sections was surrounding murine pancreas as shown by amylase immunoreactivity (Figure 3-7, 3-8, 3-9), human grafts had been depleted of exocrine tissue through pre-transplant culture and were easily identified by staining for CK7 (Figure 3-7, 3-8, 3-9). Furthermore, the human ductal cells were organized into small, tubular structures *in vivo* and these cells in general exhibited a different morphology as compared to surrounding murine pancreas.

As mentioned, human grafts at all timepoints remained primarily ductal in phenotype with cells organized into tubular structures having distinct lumens. Interestingly, although these grafts were <1% β-cells before transplant, by 25 days post-transplant numerous insulin-expressing cells were observed within ngn3 transduced grafts (Figure 3-7 E). Furthermore, a large proportion of transduced grafted cells (30-

40%) expressed the neuroendocrine protein synaptophysin (Figure 3-7 G). Neither insulin nor synaptophysin expression was observed in non-infected control grafts at the same timepoint (Figure 3-7 F, H). Analysis at later timepoints showed the same pattern, although the highest proportion of insulin-expressing cells was observed in the day 25 graft. Day 45 (Figure 3-8) and day 65 (Figure 3-9) grafts were found to contain synaptophysin-expressing cells as well as scattered insulin-positive cells, indicating full differentiation of some ngn3 transduced ductal cells to β-cell phenotype. This effect was not seen in corresponding non-infected grafts at any timepoint analyzed. Attempts to remove grafts later than 100 days post-transplant met with little success, likely due to decreased long-term graft survival.

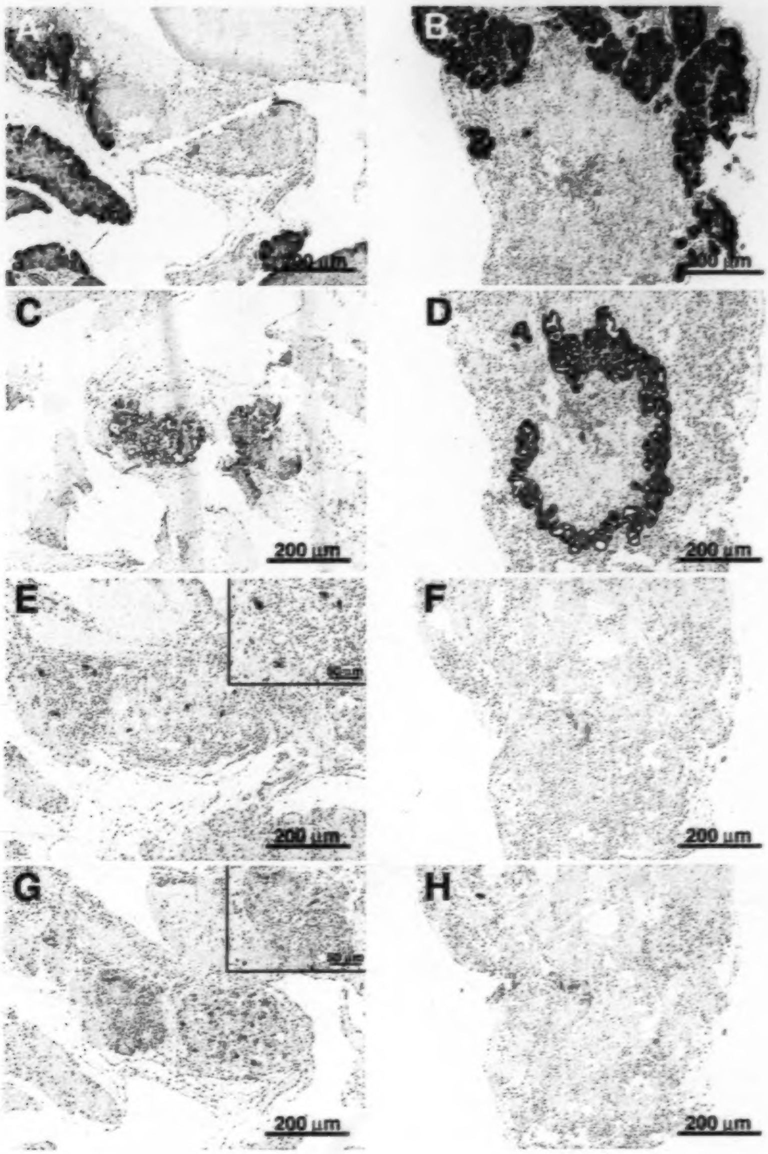
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Figure 3-7: Histological analysis of intra-pancreatic human ductal grafts removed at day 25 post-transplant. **(A, C, E, G)**: ngn3 infected graft. **(B, D, F, H)**: control non-infected graft. Grafts were immunostained for amylase **(A, B)**, CK7 **(C, D)**, insulin **(E, F)**, and synaptophysin **(G, H)** using the ABC-DAB indirect method (brown color = positive). Insets show high magnification of areas within the graft that are positive for the marker analyzed. Counterstaining of tissue sections done with hematoxylin.

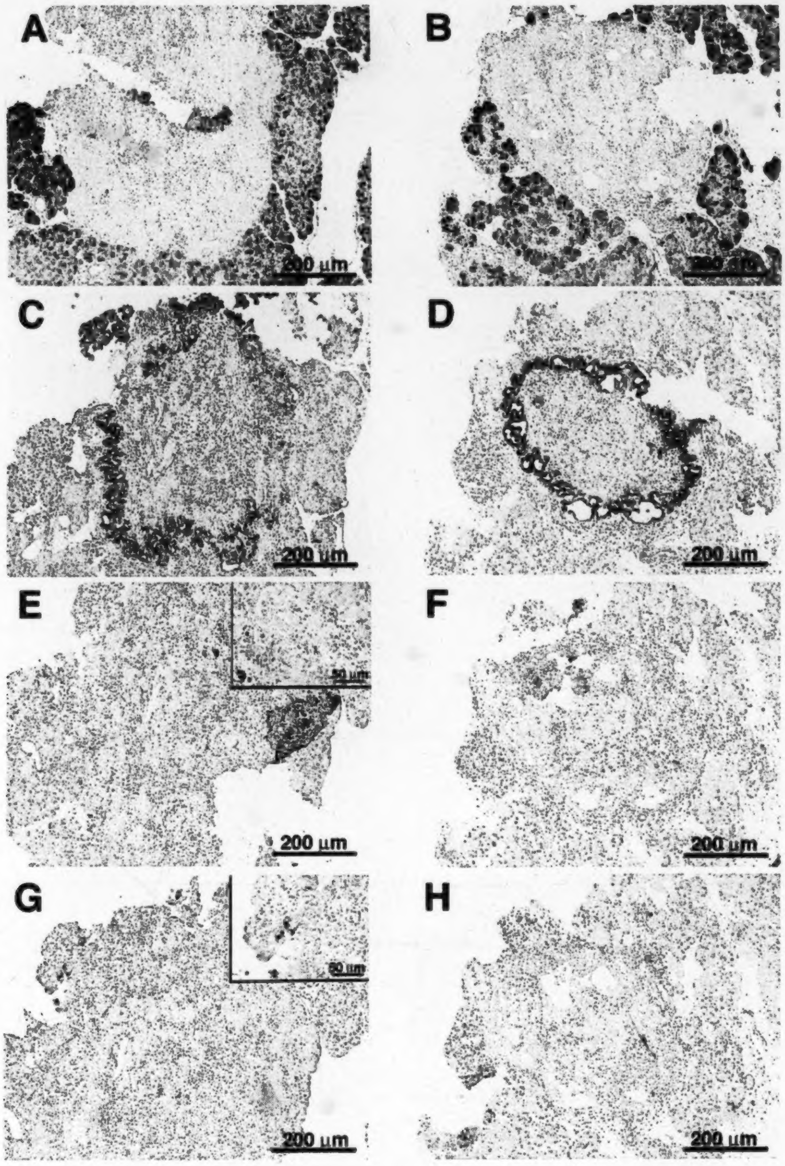
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Figure 3-8: Histological analysis of intra-pancreatic human ductal grafts removed at day 43 post-transplant. **(A, C, E, G)**: ngn3 infected graft. **(B, D, F, H)**: control non-infected graft. Grafts were immunostained for amylase **(A, B)**, CK7 **(C, D)**, insulin **(E, F)**, and synaptophysin **(G, H)** using the ABC-DAB indirect method (brown color = positive). Insets show high magnification of areas within the graft that are positive for the marker analyzed. Counterstaining of tissue sections done with hematoxylin.

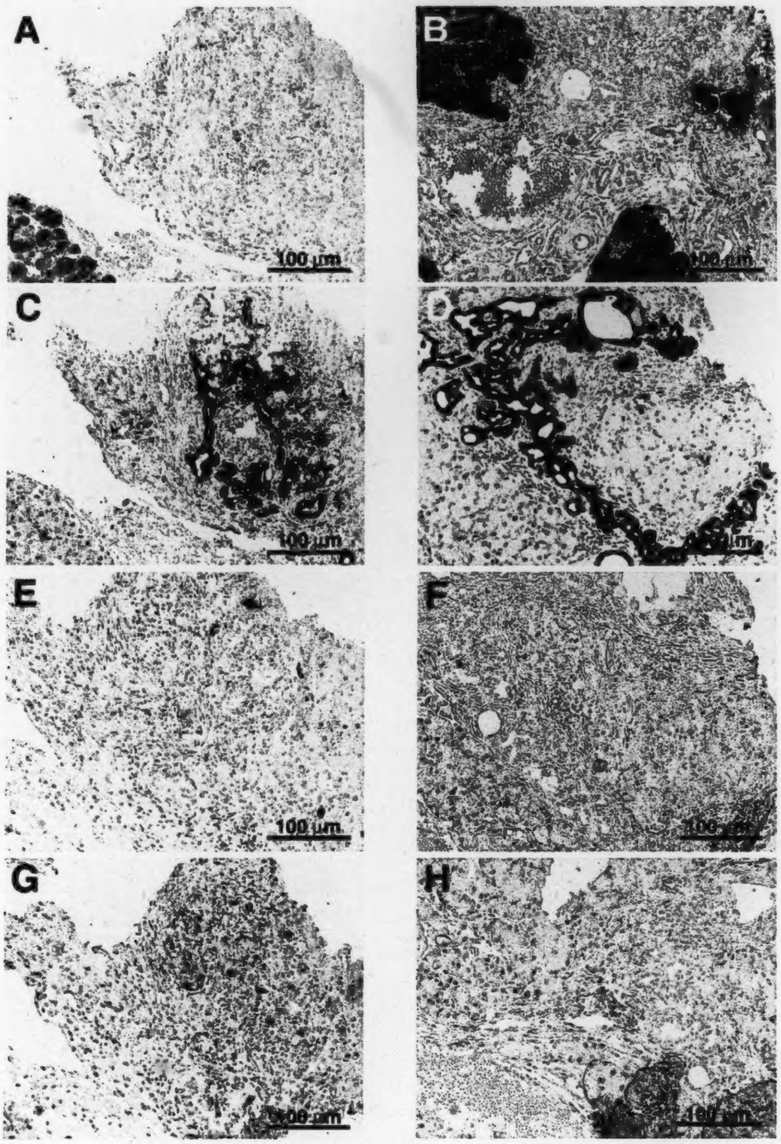
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Figure 3-9: Histological analysis of intra-pancreatic human ductal grafts removed at day 65 post-transplant. **(A, C, E, G)**: ngn3 infected graft. **(B, D, F, H)**: control non-infected graft. Grafts were immunostained for amylase **(A, B)**, CK7 **(C, D)**, insulin **(E, F)**, and synaptophysin **(G, H)** using the ABC-DAB indirect method (brown color = positive). Counterstaining of tissue sections done with hematoxylin.

Double immunofluorescent staining was performed on grafts found to contain insulin-positive cells to confirm their origin as both human and ductal. Figure 3-10 A, B shows CK7 staining in red (Cy3) and insulin in green (FITC) in two ngn3-transduced grafts. It can be seen that in most cases insulin-positive cells co-expressed CK7 and resembled "transitional cells" as described in other studies (8). To further confirm the human origin of these cells, double staining was also performed using a 100% human specific antibody to c-peptide (Figure 3-10 C). In preliminary experiments this antibody was confirmed to have no cross-reactivity with mouse c-peptide (data not shown). This analysis confirmed the presence of beta-like cells within the graft that were closely associated with ductal structures and of donor (human) origin. Finally, figure 3-10 D shows the co-localization of CK7 and synaptophysin throughout an ngn3 transduced graft, giving further evidence of transitional differentiating cells *in vivo.*

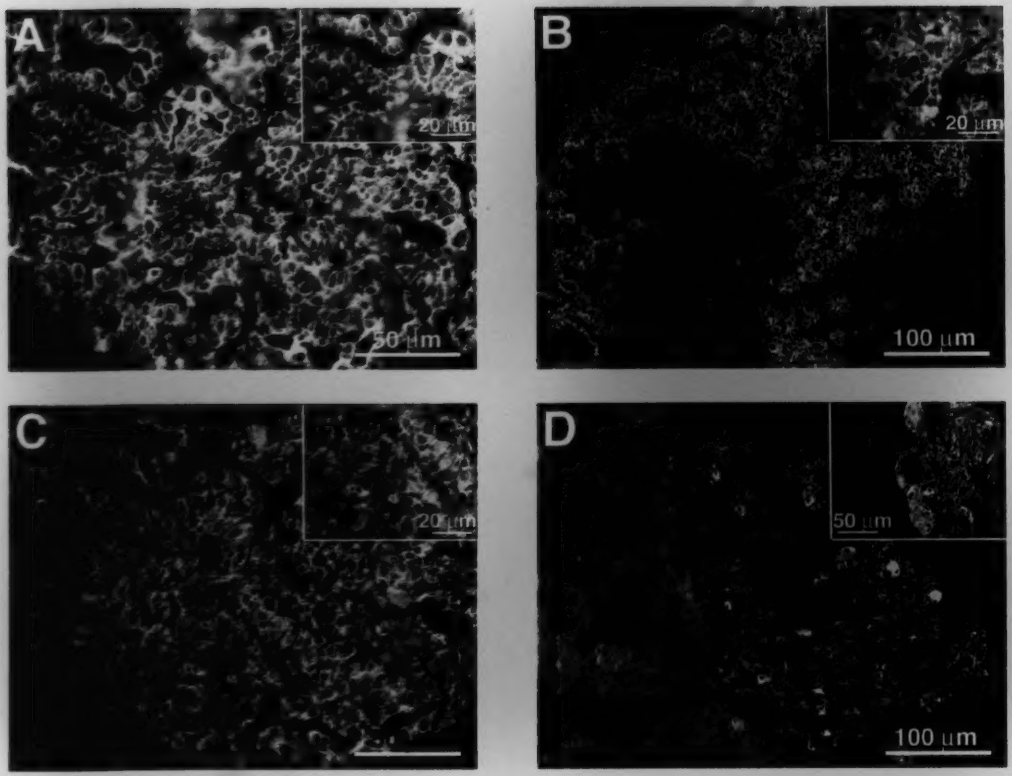

Figure 3-10: Double immunofluorescent staining of intra-pancreatic ngn3-infected human ductal grafts. **(A, B)**: Staining for CK7 in red (Cy3) and insulin in green (FITC) shows co-localization and existence of "transitional cells". **(C)**: Staining for CK7 in green (FITC) and human c-peptide in red (Cy3) confirms these beta-like cells to be of donor origin). **(D)**: Staining for CK7 in green (FITC) and synaptophysin in red (Cy3) shows extensive co-localization in transduced grafts. Insets are high magnification of areas within the grafts where co-localization of markers occurs.

Semi-quantitative RT-PCR analysis was performed on several grafts in order to confirm expression of human-specific genes. Human grafts were removed as described in the *Materials and Methods* section and trimmed of excess mouse pancreatic tissue or fat. A piece of the same mouse pancreas was also removed at this time from an area of the organ distal to the original graft site to act as a negative control for human-specific gene expression. Figure 3-11 shows representative RT-PCR results for a graft removed at day 17 post-transplant. The graft sample (lane 1) showed gene expression of human β-2 microglobulin (ubiquitously expressed in human cells) as well as human insulin, confirming the presence of human tissue and the insulin expression seen using immunostaining. In contrast, the pancreas fragment taken from an area far from the graft site was negative for both human-specific genes (lane 2). Species specificity of the primers used for RT-PCR was confirmed by also running samples from a human islet preparation (lane 3), mouse islet preparation (lane 4), and intact naïve mouse pancreas fragment (lane 5). GAPDH was used as a non-species specific marker to confirm RNA/cDNA integrity and an overall negative control was H_20 substituted for cDNA in the PCR reaction (lane 6).

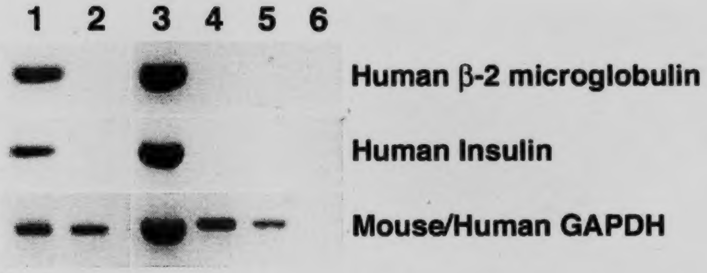

Figure 3-11: RT-PCR analysis of human specific gene expression in a transduced ductal cell graft removed at day 17 post-transplant. Samples from the removed graft (lane 1), a pancreas fragment from an area far from the transplant site (lane 2), a human islet preparation (lane 3), a mouse islet preparation (lane 4), and a pancreas fragment from a naïve mouse were assessed for gene expression of human β-2 microglobulin, human insulin, and non-species specific GAPDH. H_20 was used in place of cDNA as a negative control (lane 6).

In order to determine whether the increase in insulin-positive cells seen was due to differentiation from ductal cells or to proliferation of the small number of existing β-cells pre-transplant, immunostaining was done for the cell cycle marker Ki67. The absence of Ki67 staining in grafts containing insulin-positive cells indicated that these cells likely resulted from neogenesis and not β-cell replication.

Another set of experiments was designed to determine whether the differentiation observed *in vivo* was dependent on graft site or if it could be replicated in an extra-pancreatic location. In these experiments, infected and control tissue was transplanted into the pancreas as in the previous experiments, with a third group of animals receiving infected tissue from the same preparation under the right renal capsule opposite from the Balb/c islet graft in the left kidney. Although in controls differentiation to insulin-producing phenotype was again observed in infected grafts transplanted into the pancreas, no insulin-positive cells were seen in non-infected pancreatic grafts or in infected kidney grafts.

III-D) DISCUSSION

The production of a clinically significant number of islet endocrine cells from progenitor cells of the adult human pancreas would represent a significant advancement in the field of islet transplantation. Several groups (7, 18) have reported limited β-cell differentiation from human ductal populations, suggesting that ductal cells are adult islet progenitors. In this study, we have tested the protocol developed by Heremans *et al.* (18) and confirmed that induction of exogenous ngn3 can at least initiate β-cell differentiation in human ductal cells *in vitro*. Furthermore, we have tested a novel *in vivo* protocol for the differentiation of ngn3 expressing ductal cells and demonstrated full differentiation to an insulin-producing phenotype.

It has been well established that expression of the bHLH transcription factor ngn3 is an integral step in islet cell neogenesis during pancreatic development (20-25). In 2002, Heremans *et al.* reported that human ductal cells adenovirally transduced with murine ngn3 initiate transcription of several β-cell specific genes including insulin itself (18). Specifically, ectopic ngn3 induced transcription of downstream genes including neuroD, Pax4, and Nkx6.1. It also directed 22% of the cell population to express the neuroendocrine marker synaptophysin and 13% to express insulin, indicating differentiation to β-cell phenotype.

The first section of this study was designed to repeat the protocol of Heremans *et al.* and to assess differentiation potential in human ductal cultures from a different center. Experiments were replicated as accurately as possible, and infections were carried out using the identical adenoviral construct. As a result, we obtained very similar results with some notable exceptions. Most importantly, we did not observe an increase in the proportion of insulin-positive cells in any preparation tested. This is in contrast to the Heremans study and indicates a lack of full differentiation along the β-cell lineage. The reasons for this discrepancy are unknown, although the fact that we obtained only a 10-20% infection efficiency as compared to the 30-40% reported by the Belgian group may be a contributing factor. Despite this, our results do reflect some degree of islet differentiation and are similar to other aspects of the Heremans study. A significant increase in the proportion of cells expressing synaptophysin was observed in infected, but not control, cultures at 48 hours and 10 days post-infection. Synaptophysin is a synaptic vesicle protein expressed in differentiated neuroendocrine cells, and is involved in neurotransmitter/hormone release. Furthermore, an induction or upregulation of several downstream transcription factors from ngn3 in the pathway of islet development was observed. NeuroD appeared to be upregulated in infected cultures, and Pax4, which was not detectable in pre-infection or control samples, was induced by exogenous expression of ngn3. This has also been demonstrated in another study (29), where it was demonstrated that exogenous ngn3 expression in pancreatic ductal cell lines is sufficient to activate Pax4 expression.

Interestingly, results of this study also indicate the induction of the human version of ngn3 in cultures transduced with the murine ngn3 gene. The fact that we specifically detected human ngn3 only in infected cultures suggests that the mouse transgene was able to regulate expression of the corresponding human gene. It would be of interest to determine whether the transduced gene itself drives differentiation in these cells, or if it acts in an indirect manner to activate the human gene, which then drives the differentiation process. These results may be at odds with a recent study (30) suggesting that ngn3 uses negative feedback to repress its own expression. It would follow from this data that transduction with exogenous ngn3 should repress endogenous expression of the gene. This does not appear to be the case in our experiments with murine ngn3 experiments in human cells. Taken together with increased synaptophysin expression and induction of other transcription factors, our results indicate the partial (but not full) differentiation of ngn3 expressing human ductal cells to β-cell phenotype *in vitro*. Further experiments are necessary to determine whether modification of culture conditions can drive further differentiation in transduced cultures.

Because full differentiation to insulin-producing phenotype was not accomplished under *in vitro* conditions, further experiments were designed to test the potential for *in vivo* differentiation of transduced human ductal tissue. It was hypothesized that an *in vivo* environment may provide more suitable conditions for differentiation with respect to growth factors, support matrices, and nutrient supply. Specifically, the pancreas was chosen as the site for engraftment. In addition, it was hypothesized that a model of islet injury would be necessary to create the stimulus for differentiation and restoration of insulin levels. To this end, we assessed transduced graft differentiation in immunocompromised mice rendered hyperglycemic with STZ and subsequently normalized with a small mouse islet graft to ensure graft and animal survival.

Following confirmation of initial engraftment and *in vivo* transgene expression, differentiation was assessed at timepoints up to 100 days post-transplant. A marked difference in immunostaining of transduced and control grafts was observed in most cases. Ngn3 expressing grafts generally stained strongly for synaptophysin and contained distinct insulin-positive cells. This was in contrast to control grafts, which did not contain insulin-expressing cells and seldom stained positive for synaptophysin. This indicates that transduced cells are able to "activate" the pathway toward β-cell differentiation when placed in a suitable environment and that *in vivo* conditions may be more supportive of this process than *in vitro*. The fact that these differentiated cells did not express the proliferation marker Ki67 provides evidence that the increase in insulin-positive cells is due to neogenesis from ductal precursors and not to existing β-cell replication. Moreover, the numerous "transitional cells" expressing both ductal and β-cell markers observed in infected grafts suggests neogenesis over proliferation as a mechanism for increased insulin-positivity.

Although neogenesis from the native pancreas has been reported in several models of pancreatic injury (31-34), to our knowledge this is the first report of *in vivo* differentiation of engrafted genetically modified adult human ductal cell to insulin-producing phenotype. Gao *et al.* (35) reported poor engraftment and limited viability of adult human ductal tissue transplanted into nude mice, with little evidence of differentiation. Significantly, cultures used in that study were taken from less dense Ficoll gradient layers after purification and likely contained numerous endocrine cells to begin with. In contrast, our experiments utilized tissue only from the most dense exocrine pellet layer after Ficoll purification which were carefully characterized to contain <1% β-cells after culture. We consider this to be an important aspect in these differentiation studies, as "contaminating" β-cells present may confound results due to de-differentiation in culture followed by subsequent re-differentiation of existing β-cells under the appropriate stimuli. This effect could easily be mistaken as true neogenesis from precursor cells and must be carefully controlled. It should also be noted that even in cultures of <1% β-cells, insulin mRNA is still strongly detected at all timepoints using sensitive methods such as RT-PCR, and this should not be misinterpreted as evidence of β-cell differentiation.

Transplant site is an important consideration in islet transplantation, as different tissues provide advantages or disadvantages with respect to vascularization, graft survival, and overall effectiveness. In differentiation studies, it is also relevant to determine whether a specific grafting site can provide a suitable milieu for islet neogenesis. We chose the pancreas as a novel site for transplantation of ngn3 expressing human ductal cells. Although technically difficult compared to other sites (ie. kidney, liver), it was hypothesized that a damaged pancreas would provide ideal support for islet neogenesis through exposure to the proper concentration and combination of growth factors. As predicted, the differentiation seen in these experiments was a site-specific effect and was not reproduced in grafts placed under the renal capsule. In addition, significant vascularization was observed in intra-pancreatic grafts, likely facilitating survival and growth factor/cytokine delivery at this site. In our hands, regeneration of the native pancreas was not observed in response to STZ treatment in these adult animals and insulin staining within the graft was confirmed to be in cells of human origin.

In conclusion, this study has demonstrated the ability of adult human ductal cultures induced to express exogenous ngn3 to differentiate to insulin producing phenotype. Although only partial differentiation was seen under *in vitro* conditions, transplantation of transduced tissue into the damaged pancreas was able to drive the process to completion. The results of this study are promising, however two major limitations exist. Firstly, infection efficiency in these cells using adenoviral delivery is relatively low. This is most likely due to the size and nature of cellular aggregates present and inability of the virus to penetrate into all cells. Further experiments are currently underway to fully dissociate these aggregates prior to infection and subsequently re-aggregate in suspension culture prior to transplant to increase viral exposure to all cells. Secondly, the degree of differentiation in these experiments was in no case sufficient to sustain normoglycemia in diabetic animals after removal of the supporting islet graft. We believe this to also be a problem primarily with infection efficiency and that better transduction will result in greater levels of differentiation and insulin production *in vivo*. Despite these limitations, this study provides further evidence that non-endocrine adult pancreatic tissue is a promising potential source of transplantable islets for type 1 diabetes.

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CHAPTER IV

ISOLATION OF CD44 AND TRK-A POSITIVE CELL POPULATIONS IN HUMAN PANCREATA AND ENRICHED PANCREATIC DUCTAL CULTURES

IV-A) INTRODUCTION

In order to build on recent successes in the field of islet transplantation for type 1 diabetes (1), an alternative source of insulin-producing tissue must be identified to supplement current limited supplies. One attractive possibility is the identification and exploitation of islet progenitor cells or stem cells in the adult human pancreas.

It has been well established that islet turnover in the mature pancreas occurs via cells that reside in or near the ductal epithelium (2-4). Furthermore, both *in vitro* (5-8) and *in vivo* (9, 10) models have been used to demonstrate the neogenesis of islet β-cells from pancreatic ductal cell populations. However, despite the evidence to date a specific phenotypic islet progenitor cell has not yet been described. For this to occur, specific cellular markers must be identified so that an islet progenitor or stem cell can be first recognized and then separated from surrounding pancreatic ductal tissue. In order to narrow the search for these precursors, it may be useful to look to other, more extensively characterized tissue model systems.

Adult stem cells have been identified in tissues such as muscle (11), central nervous system (12), and blood (13) and have been isolated based on expression of characteristic cell surface proteins. Trk-A, the receptor for nerve growth factor (NGF), for example, has been shown to be expressed in neurosphere-derived stem cells and is an effector of neural differentiation (14). In addition, this receptor is also expressed on hepatic stellate cells (15), which are involved in liver remodeling and regeneration. More recently, Trk-A has been shown to be expressed on primary pancreatic cells as well as pancreatic exocrine AR42J cells (16), a cell line capable of differentiation into insulin-producing phenotype (17). Moreover, it is also known that Trk-A is expressed on ductal-epithelial cells of the embryonic and fetal rat pancreas at specific developmental stages (18), and that islet morphogenesis is reduced in the presence of agents that reduce tyrosine kinase activity in this receptor (19). These observations suggest that cells expressing Trk-A in the mature pancreas may play a role in islet regeneration. It is yet to be demonstrated whether a sub-population of Trk-A-positive cells can be isolated from the adult pancreas and what significance they have in islet neogenesis.

Other receptors or cell surface proteins may also be potential markers to identify and isolate pancreatic stem cells. The hyaluronan receptor CD44 (HCAM) is a glycoprotein molecule involved in cell attachment and cell-cell interactions (20). It is expressed in normal pancreatic ducts (21, 22), and is upregulated in metaplastic ductal cell adenocarcinomas (23). Furthermore, it has been proposed that CD44 expression in hyperplastic ductal clusters is an indicator of de-differentiation. CD44 expressing ductal cells, therefore, are another potential mature pancreatic progenitor population.

The isolation of a sub-population of pancreatic cells capable of uniform islet endocrine neogenesis would be a significant step in the production of an abundant, homogenous supply of insulin-producing tissue. In this study, expression patterns of the cell surface receptors Trk-A and CD44 were characterized in intact human pancreatic biopsies. FACS and immunostaining were then used to identify and isolate sub-populations expressing these markers from enriched ductal cultures derived through culture of non-endocrine pancreatic digest. Sorted sub-populations were subsequently analyzed using immunohistochemistry, semi-quantitative RT-PCR, and quantitative real-time RT-PCR to define differences in gene expression indicative of the potential for islet neogenesis. Finally Trk-A sorted cells were cultured as monolayers and exposed to NGF to study the phenotypic differences between positive and negative populations with and without receptor (NGF) stimulation.

IV-B) MATERIALS AND METHODS

1. Tissue Procurement and Culture

All experimental protocols used in this study were approved by the Research Ethics Board of the University of Alberta. Human donor pancreases were removed from cadaveric donors with informed research consent from relatives and processed according to the protocols described by this laboratory (1, 24). Before collagenase digestion of the pancreas, two biopsies were dissected from the head region of the organ and fixed in Z-fix (Anatech Ltd. Battle Creek, MI) for subsequent immunohistochemical anlysis. For isolation of pancreatic islets, organs were cold stored in University of Wisconsin (U.W.) solution and perfused via the duct with the enzyme solution Liberase (Roche, Indianapolis, USA). Once digested, islets were purified on continuous Ficoll gradients using the refrigerated Cobe 2991 (COBE BCT inc., Lakewood, USA) blood cell processor. Immediately following density gradient purification, the predominately acinar cell fraction was collected from the Cobe bag and washed three times with Hanks balanced salt solution (HBSS; Sigma-Aldrich, Oakville, Canada) supplemented with

0.5% bovine serum albumin (BSA; fraction V, Sigma). After dithizone staining of representative samples to assess islet content, tissue was cultured in suspension with approximately 500 μl "pack tissue volume" per non-treated 15 cm plate (Fisher Scientific, Edmonton, Canada) in RPMI 1640 medium (Gibco/Invitrogen, Burlington, Canada) supplemented with 1% insulin-transferrin-selenium (ITS) and 0.5% human serum albumin (HSA) (all supplements/sera from Sigma). Media changes were performed at day 1 and day 3 post-culture. This serum-free protocol has been previously confirmed to result in an enriched population of ductal-epithelial cells after 4 day culture (25) that was subsequently used for FACS analysis.

2. Flow Cytometry and Cell Sorting

Cellular aggregates were dissociated by shaking in a 37°C water bath in Ca^{2+}-free media supplemented with 1 mM EGTA and 0.5% BSA for 7 minutes before addition of trypsin (25 μg/ml) and DNAse (4 μg/ml) and further agitation for 12 minutes. Single cells were then separated by centrifugation from non-dissociated aggregates and the process was repeated to obtain a complete single cell preparation. For CD44 staining, single cells were incubated with 1/10 mouse anti-human FITC-conjugated CD44 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at 4°C. Cells were then washed 2 times with Ca^{2+}-free media and re-suspended in the same media supplemented with 10 μl/ml DNAse to prevent clumping. Subsequently, cells were sorted on an EPICSAltra (Beckmann Coulter Canada inc., Mississauga, Canada) using viability and fluorescence as positive gates as compared to isotype control (1/10 FITC conjugated IgG1). Alternatively, for Trk-A staining, cells were indirectly stained with 1/50 goat anti-human Trk-A antibody (R&D Systems, Minneapolis, MN) for 30 minutes at 4°C.

Secondary antibody incubation was for 30 minutes at 4°C using 1/25 FITC-conjugated Affinipure rabbit anti-goat IgG (Jackson Immunoresearch Laboratories inc., Westgrove, PA). Single cells were then washed, resuspended as described above, and sorted using secondary antibody alone to determine the positive fluorescent gate. Negative cell populations were also sorted for comparison.

3. Post-FACS culture of Trk-A sorted populations

Immediately post-FACS, two preparations sorted on the basis of Trk-A expression were re-cultured in RPMI supplemented with 1% ITS and 0.5% HSA in 4-well Biocoat collagen-coated chamber slides (Becton-Dickinson Labware, Bedford, MA). Cells were seeded in 1 ml of media at approximately 0.5 million cells per well. For both Trk-A positive and negative populations, some wells were supplemented with 100 ng/ml NGF (Sigma) and some were left as negative controls for growth factor treatment. All chamber slide cultures were incubated at 37 °C for ~24 hours until the cells had adhered to the culture surface, after which fresh media and NGF was added immediately and at 2 day intervals for the remainder of the culture period. After 5-6 days in culture the slides were first subjected to static incubation to assess insulin release, and then washed and fixed in Z-fix (Anatech Ltd., Battle Creek, MI) for histological analysis.

4. Immunohistochemistry

For determination of cellular composition before and after sorting, aggregates were dissociated into single cell suspensions to facilitate quantification of immunostaining. Single cell suspensions were obtained by mechanical disruption using pipetting at 37°C in Ca^{2+}-free media supplemented with 1 mM EGTA and 0.5% BSA for 7 minutes before addition of trypsin (25 μg/ml) and DNAse (4 μg/ml) and further pipetting for 4 minutes. Cells were then allowed to adhere to histobond slides (Marienfeld, Germany) and fixed in Bouin's fixative for 12 minutes before storage at 4°C in 70% ethanol.

Immunostaining of single cell preparations, pancreatic biopsies, and chamber slide cultures was performed using the ABC-DAB method (Avidin-Biotin complex visualized with the chromagen diaminobenzidine). Sections or cells were quenched with a 20% H_2O_2/80% methanol solution. Microwave antigen retrieval for CD44, cytokeratin 7 (CK7), and Trk-A staining involved: 15 minutes on high power (Sanyo household model, 1260W) in 800 ml Na^+ citrate for tissue sections and 5 seconds on high 6 times in 40 ml sodium citrate for single cells. Blocking was performed with 20% normal goat serum (Fisher) for 15 minutes. Primary antibody concentrations were as follows: 1/50 mouse anti-human CD44 (Santa Cruz), 1/1000 guinea pig anti-porcine insulin (Dako Diagnostics Canada inc., Mississauga, Canada), 1/200 mouse anti-human CK7 (Dako), 1/100 mouse anti-human vimentin (Dako), and 1/100 rabbit anti-human Trk-A (Santa Cruz). Primary antibody incubations were 30 minutes at room temperature followed by wash (3X) with PBS before addition of secondary antibody. All biotinylated secondary antibodies were obtained from Vector Laboratories (Burlingame, USA) and used at a concentration of 1/200 for 20 minutes. ABC complex (Vector) incubation time was 40 minutes and visualization was with the chromagen diaminobenzidine (Biogenex, San Ramon USA) for 5 minutes. Indirect double immunofluorescence was performed sequentially with goat anti-mouse FITC, goat anti-rabbit Cy3, and goat anti-guinea pig FITC conjugated secondary antibodies (all from Jackson ImmunoResearch Laboratories inc., Pennsylvania, USA) used at 1/200. All images were captured on a Zeiss Axioskop II fluorescent microscope (Carl Zeiss Canada) with a Coolsnap camera and IP lab software (Scanalytics inc.). Single cell counts were performed on a minimum of 500 cells per sample and percentages calculated as number of positive per 500. Negative controls were performed for all immunostaining procedures and consisted of the above protocol with elimination of the primary antibody (not included in figures).

5. Semi-quantitative RT-PCR analysis

Samples from sorted cell populations were suspended in Trizol reagent (Invitrogen, Carlsbad, CA) and stored at –80°C for molecular analysis. Samples were subsequently thawed and RNA extracted according to manufacturers protocol (Invitrogen). cDNA was constructed from 1 µg total RNA with 1.25 units per µl of Multiscribe reverse transciptase in 1X Taqman RT buffer containing 5.5 mM $MgCl_2$, 500 µM dNTPs, 2.5 µM oligo dT16, and 0.4 U/µl RNAse inhibitor in a total volume of 50 µl. For RT-PCR, 1 µl of cDNA was used per 25 µl reaction along with 1X PCR buffer, 2 mM MgCl, 0.2 mM dNTPs, 1.5 units of taq polymerase (5 U/µl) (Invitrogen), and 0.3 µM of each primer pair. All chemicals/reagents unless otherwise noted were from Applied Biosystems (Foster City, CA), and previously used primer sequences were obtained from Assady *et al.* (26) (PDX-1, insulin), Zulewski *et al.* (27) (nestin, amylase), and Heremans *et al.* (8) (neuroD, CK19, glucagon, insulin). For all PCR reactions, 35 cycles were performed, with 30 sec. denaturation at 94°C, 30 sec. annealing at 58°C, and 30 sec. extension at 72°C. Final polymerization was at 72°C for 10 minutes followed by a 4°C hold. Products were separated on an ethidium bromide stained 2% agarose gel and images captured with Alpha Digidoc software (Perkin-Elmer, Boston, USA). Primer sequences were as follows: 5'-CCC ATG GAT GAA GTC TAC C-3' (forward) and 5'-

GTC CTC CTC CTT TTT CCA C-3' (reverse) (PDX-1-262 b.p. fragment), 5'ATC CCA ACC CAC CAC CAA CC-3' (forward) and 5'-CAG CGG TGC CTG AGA AGA TT-3' (reverse) (neuroD-439 b.p. fragment), 5'-GCA GCC TTT GTG AAC CAA CA-3' (forward) and 5'-GTT GCA GTA GTT CTC CAG GTG-3' (reverse) (insulin-263 b.p. fragment), 5'-CCC AAG ATT TTG TGC AGT GGT T-3' (forward) and 5'-GCG GCC AAG TTC TTC AAC AAT-3' (reverse) (glucagon-221 b.p. fragment), 5'-ACC TGC GTG GCT CTG TTA CT-3' (forward) and 5'-CAG CGT GTC CTC TTT GTG TC-3' (reverse) (pancreatic polypeptide-180 b.p. fragment), 5'-TTT GTC ACC GTG GCC GTG TTT-3' (forward) and 5'-TTG CAT GTG TTC CCT GTC TGG-3' (reverse) (synaptophysin-253 b.p. fragment), 5'-ATG AAC GGG CTG GAG CAG TCT GAG GAA AGT-3' (forward) and 5'-CTG AGG ACC AGG ACT CTC TA-3' (reverse) (nestin-191 b.p. fragment), 5'-GAC TTT CCA GCA GTC CCA TA-3' (forward) and 5'-GTT TAC TTC CTG CAG GGA AC-3' (reverse) (pancreatic amylase-300 b.p. fragment), 5'-CTT TTC GCG CGC CCA GCA TT-3' (forward) and 5'-GAT CTT CCT GTC CCT CGA GC-3' (reverse) (CK19-1050 b.p. fragment), 5'-CCA GCA GAG AAT GGA AAG TC-3' (forward) and 5'-GAT GCT GCT TAC ATG TCT CG-3' (reverse) (β-2 microglobulin-268 b.p. fragment). β-2 microglobulin primers were used to verify the absence of genomic DNA contamination in samples as only the 268 b.p. fragment was seen and not the 900 b.p. genomic version of the gene. Furthermore, all primers used except those for nestin and neuroD spanned at least one intron. A previously characterized enriched human islet preparation was used as a positive control for several genes examined as well as for comparison with human ductal cultures. Positive controls for transiently expressed developmental factors were samples from previous experiments found to contain the mRNA for these genes. All products were verified through sequencing using a TOPO TA cloning kit (Invitrogen) and BLAST search against known GenBank sequences (Accession numbers: NM_004048 for β-2 microglobulin, NM_000207 for insulin, X03991 for glucagon, NM_002722 for pancreatic polypeptide, NM_002500 for neuroD, NM_000209 for PDX-1, NM-003179 for synaptophysin, X65964.1 for nestin, NM_020978 for amylase, NM_002276 for CK19). Negative controls consisted of both cDNA absent for the gene of interest and Sigma water (Sigma) in place of experimental cDNA.

6. Quantitative (real-time) PCR analysis

Insulin and PDX-1 mRNA expression levels were analyzed in CD44 and Trk-A sorted human ductal populations using real time RT-PCR on a ABI Prism 7000 sequence detection system (Applied Biosystems). RNA samples were extracted and cDNA was prepared as described above with cDNA stored at –20°C subsequent to real time analysis. Individual PCR reactions for each sample were performed in triplicate in MicroAmp optical 96 well reaction plates using the Taqman Universal PCR Mastermix protocol and consisted of 1.0 µl cDNA, 25 ul PCR Master mix, 19.75 ul H_20 (Sigma), 0.3 µM forward and reverse primers, and either 0.15 µM (insulin, PDX-1) or 0.133 µM (GAPDH) probe (all primers and PCR reagents were obtained from Applied Biosystems) for a final reaction volume of 50 µl. Primer-probe sequences were constructed using “Primer-express” software (Applied Biosystems) with the exception of GAPDH which was obtained commercially from Applied Biosystems (catalogue #402869; probe: Joe/Tamra fluorescent tags) and were as follows: 5’-CCT GCA GCC CTT GGC C-3’ (forward), 5’-CAG ATG CTG GTA CAG CAT TGT TC-3’ (reverse), and 5’-FamTCC CTG CAG

AAG CGT GGC ATT GTamra-3' (probe) (insulin-71 b.p. fragment); 5'-ACA AGC GGA CGC GCA-3' (forward), 5'-CGT GAG ATG TAC TTG TTG AAT AGG AAC-3' (reverse), and 5'-FamCAC GCG CGC ACA GCT GCT AGATamra-3' (probe) (PDX-1-81 b.p. fragment). Cycling conditions consisted of an initial incubation at 50°C for 2 minutes then 95°C for 10 minutes followed by 40 cycles alternating between 95°C for 15 seconds and 60°C for 1 minute. Results were calculated on Microsoft Excel software using a comparative threshold method (ΔΔCt) with a standard curve according to Perkin Elmer ABI Prism 7700 User Bulletin #2, 1997. Briefly, data was collected for cycle threshold (Ct) at which the fluorescent signal of the experimental primer/probe set appears above background and normalized using the Ct of the housekeeping gene GAPDH in the same sample (GAPDH and experimental gene were run in separate wells). The resulting ΔCt was compared to a previously characterized human islet preparation sample to calculate a ΔΔCt and finally expressed as 2(-ΔΔCt) for comparison of mRNA expression between samples. All primer/probe calibration curves showed strong linear correlations (>0.98).

7. Static Incubation

Glucose stimulated insulin release from cultured Trk-A positive and negative populations was performed by removing all culture media from the chamber slide wells and replacing with 1 ml of Hams-F10 (Gibco) supplemented with either 2.8 mM or 20 mM d-glucose. Incubation in this media was at 37 °C and after 2 hours and 24 hours 500 μl of supernatant was removed into glass tubes (Becton-Dickenson), frozen, and subsequently assayed by radioimmunoassay as previously described (28) for insulin content.

8. Statistical Analysis

Experimental values for cell composition and sorted populations are expressed as a mean ± standard deviation. Statistical significance was calculated using ANOVA with post-hoc Scheffe's test using Statview (SAS Institute Inc, Cary, NC). All significance levels were set at $p<0.05$.

IV-C) RESULTS

1. CD44 expression in the adult human pancreas

CD44 expression was assessed by immunostaining in human pancreatic biopsies taken prior to collagenase digestion and islet isolation. Expression was found to be limited and sections from some donors showed no staining for CD44. In certain sections, however, small focal areas of CD44-expressing cells were observed (Figure 4-1), and these cells often appeared to be organized into ductal-like structures. Similarly, when the proportion of CD44-positive cells was assessed in dissociated pancreatic digest immediately post-islet isolation ($n = 5$), a very low level of expression was observed (0.2±0.1%), confirming that few cells express the receptor in the adult human pancreas.

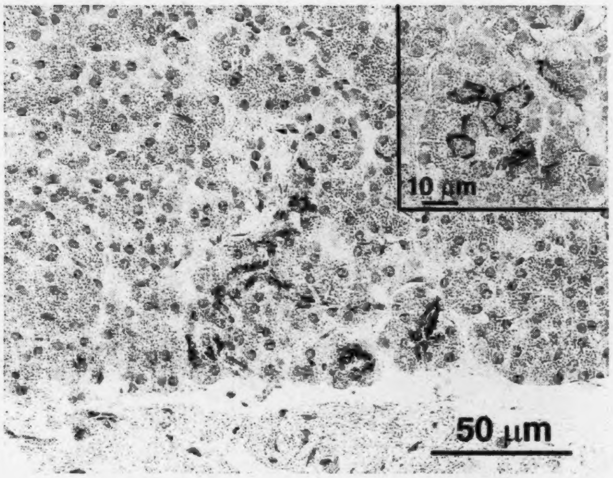


Figure 4-1: Immunostaining of a human pancreatic biopsy to detect expression of CD44 (brown colour). Inset shows a focal area of CD44 expressing cells organized into a ductal-like structure.

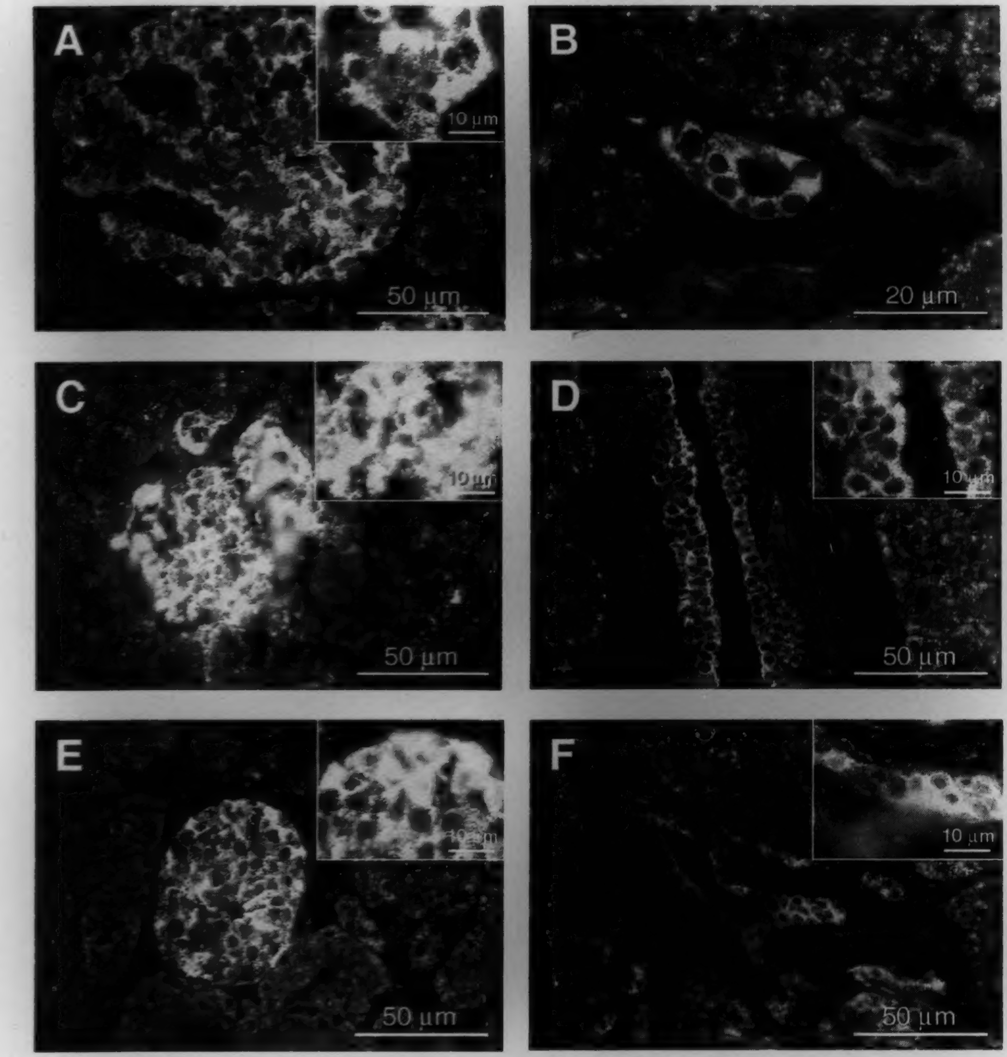
2. CD44 expression increases in cultured human pancreatic tissue

Non-endocrine human pancreatic digest was cultured for 4 days according to methods described by this lab (25) and others (29, 30) resulting in an enriched ductal cell population (86.7±8.5% CK7 positive, 9.5±10.5% amylase positive, <1% insulin positive). These populations were assessed using immunostaining and FACS for CD44 expression. Surprisingly, immunostaining at day 4 culture demonstrated that 36.1±20.0% of the cells present now expressed the receptor. This was significantly higher than the proportion of CD44-positive cells present prior to culture (36.1±20.0% vs. 0.2±0.1%; $p<0.0005$). This difference may be due to an induction of CD44 expression on non-expressing cells over the culture period. Alternatively, extensive cell death in these cultures between islet isolation and day 4 culture (25) suggests that this increase may be due to the selective cell death of non-CD44 expressing cells and resulting enrichment of CD44-positive cells in culture.

FACS analysis of single cell preparations from enriched cultures confirmed the increase in CD44 positive cells over 4 days culture. In preparations subjected to FACS analysis (n = 14), 25.0±12.4% of cells were found to have some level of CD44 expression. Representative FACS results for CD44 expression in human pancreatic ductal preparations are shown in Figure 4-3 A.

3. Trk-A expression in neonatal, juvenile, and adult human pancreatic biopsies

Immunostaining was performed on pancreatic biopsies from a neonatal, juvenile (9 year old), and adult (54 year old) human pancreas to assess localization of Trk-A receptor expression. Expression was detected on islet cells from all 3 ages (Figure 4-2), with many of the Trk-A positive cells from each age group also staining positive for insulin (Figure 4-2 A, C, E). Expression was limited in the exocrine compartment in all cases, however a proportion of cells within the pancreatic ducts stained positive for Trk-A in both the young and older pancreas (Figure 4-2 B, D, F).


A
10 µm
50 µm
B
20 µm
C
10 µm
50 µm
D
10 µm
50 µm
E
10 µm
50 µm
F
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50 µm

Figure 4-2: Double immunofluorescent staining of human pancreatic biopsies from donors of different age groups to detect expression of Trk-A. Staining was performed using either insulin (FITC) + Trk-A (Cy3) to visualize islets **(A, C, E)**, or CK7 (FITC) + Trk-A (Cy3) to visualize ductal structures **(B, D, F)**. Expression is shown in neonatal human pancreatic islets **(A)** and ducts **(B)**, juvenile (9 year old) islets **(C)** and ducts **(D)**, adult (54 year old) islets **(E)**, and ducts **(F)**. Insets show high magnification of areas of Trk-A expression or co-localization in islet and duct structures.

4. Identification of a Trk-A positive population in cultured human pancreatic ductal tissue

Trk-A expression was also assessed quantitatively by immunostaining of dissociated enriched human ductal cultures 4 days post-islet isolation (n = 12). It was found that 57.4±15.6% (range: 24%-71.5%) of cells in these cultures stained positive for the receptor. Furthermore, these preparations were subdivided into donor age classes of <25 years (n =6) and >50 years (n = 6) to assess age-related differences in Trk-A expression in the ductal population. However, despite previous reports of reduced or absent expression in mature ducts (18), a statistically significant difference was not found between the proportion of Trk-A expressing ductal cells in these groups (54.9±20.9% in <25 years vs. 59.8±9.2% in >50 years; p>0.05).

FACS was also performed on human ductal preparations (n = 11) to assess expression levels and to isolate positive/negative populations for further analysis. By this method, it was found that 33.8±18.7% of ductal cells expressed Trk-A after 4 days in culture. It should be noted that differences between immunostaining and FACS results may be explained by the fact that different antibodies were used for staining fixed cells and for FACS, and that the 11 preparations assessed by FACS were not the same preparations initially assessed using immunostaining. A representative profile for FACS analysis of Trk-A expression is shown in Figure 4-3 B.

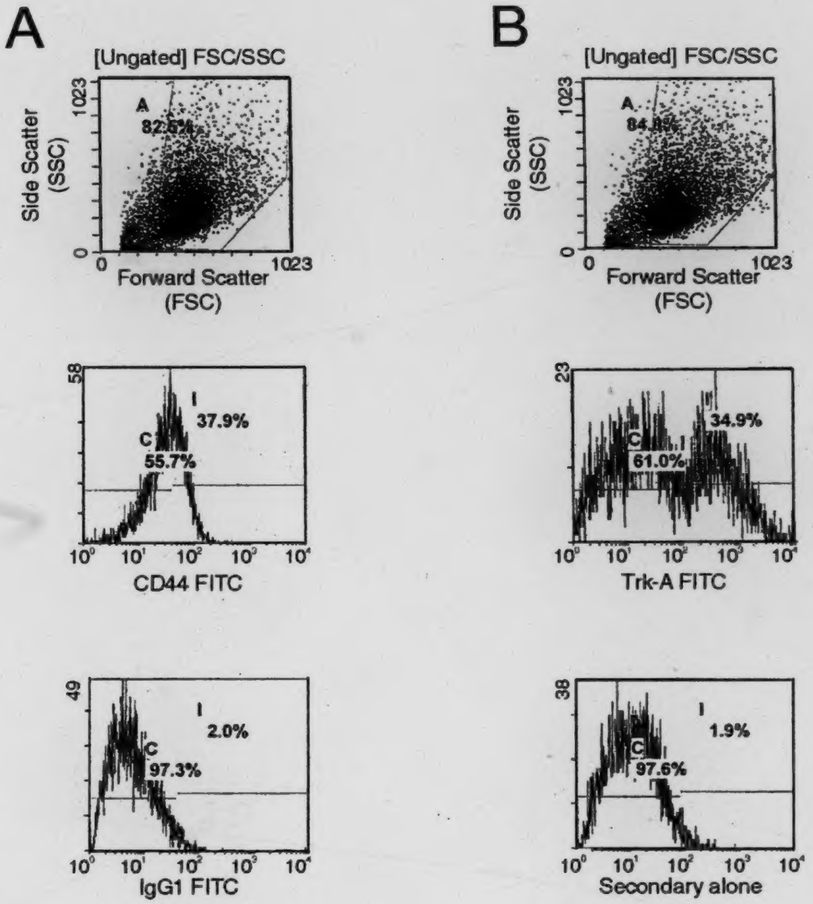

Figure 4-3: Representative results of FACS analysis of human ductal cell populations for expression of CD44 **(A)** and Trk-A **(B)**. Dot plots show forward vs. side scatter to gate for viable cells. Histograms show fluorescence in stained cell samples as well as appropriate negative control samples.

5. Assessment of gene expression differences in CD44 and Trk-A positive and negative cell populations

For samples sorted on the basis of CD44 expression, an average of $1.3\times10^6\pm0.8\times10^6$ positive cells (>95% purity) and $1.5\times10^6\pm0.7\times10^6$ negative cells (>90% purity) were collected for either RNA analysis or immunostaining. Both positive and negative populations were confirmed by immunostaining to remain primarily ductal in phenotype after sorting (79.0±10.6% post-sort vs. 86.7±8.5% pre-sort; $p>0.05$). However, as a small proportion of these preparations also consisted of surviving exocrine and islet endocrine cells, it was not surprising that in addition to CK19 transcripts, mRNA was also detected for amylase as well as the islet endocrine hormones insulin, glucagon, somatostatin, and pancreatic polypeptide (PP) using RT-PCR (Figure 4-4 A). Assessment of several proposed islet developmental markers in CD44 positive and negative populations gave variable results. While synaptophysin (a neuroendocrine marker) was detected only in the CD44 positive population, Pancreatic and Duodenal Homeobox Gene-1 (PDX-1) was detected in both CD44 positive and negative populations, and the further downstream transcription factor neuroD was only present in the CD44 negative fraction (Figure 4-4 A). The intermediate filament protein nestin was not expressed in either CD44 positive or negative samples.

Because immunostaining and RT-PCR confirmed the presence of β-cells in enriched ductal preparations, we used real-time quantitative RT-PCR to analyze sorted CD44 populations for differences in insulin gene expression as an indicator of β-cell content. Although no significant differences in insulin gene expression were observed between these populations, a small difference in PDX-1 expression was seen whereby

CD44 negative populations contained 1.5-2 fold more PDX-1 mRNA than CD44 positive populations. Interestingly, when compared to a purified human islet preparation, it was found that although the islet graft exhibited a much higher level of insulin gene expression (100-5000 fold), PDX-1 mRNA expression in CD44 negative populations was comparable to that found in the islet preparation.

For Trk-A sorted populations, an average of $1.5 \times 10^6 \pm 0.8 \times 10^6$ positive cells (>95% purity) and $1.8 \times 10^6 \pm 1.4 \times 10^6$ negative cells (>90% purity) were collected for subsequent analysis. Similar to CD44 sorted preparations, Trk-A positive and negative populations both expressed CK19, amylase, and all four islet endocrine hormones (Figure 4-4 A), indicating the presence of ductal, exocrine, and islet cells. Synaptophysin transcripts were detected weakly in only the Trk-A positive population, PDX-1 and neuroD were present in both, and nestin was expressed in neither Trk-A positive or negative populations.

Using real-time analysis of insulin and PDX-1 gene expression levels in Trk-A sorted populations, it was found that although PDX-1 expression was not significantly different between positive and negative samples, by far the greater level of insulin expression was seen in the Trk-A positive cell population (Figure 4-4 B). The level of insulin mRNA expression in a human islet preparation, however, was still 50-5000 fold more than that even in Trk-A positive ductal populations, and 400-100,000 times greater than that in Trk-A negative populations. As with PDX-1 gene expression in CD44 sorted populations, Trk-A positive and negative samples showed levels of PDX-1 mRNA comparable to that found in a human islet preparation.

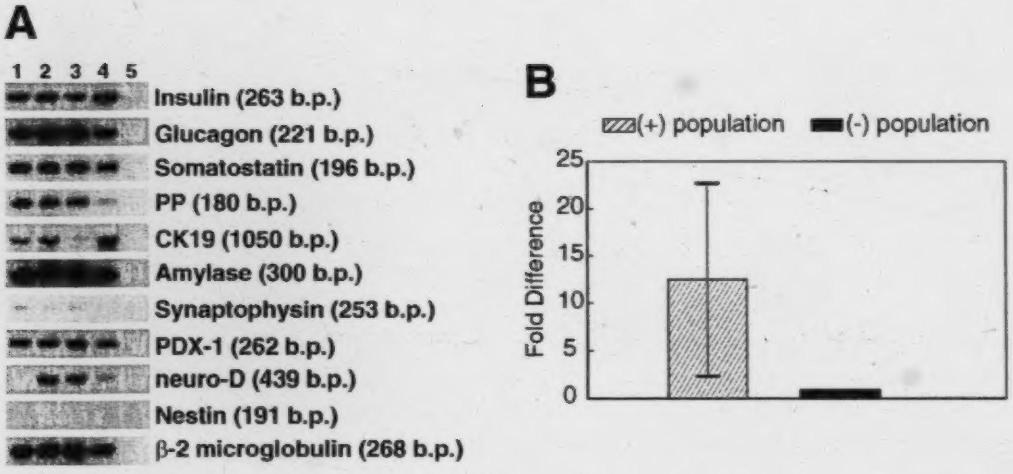

Figure 4-4: RT-PCR and real time analysis of CD44 and Trk-A positive/negative sorted cell populations. **(A)** Semi quantitative RT-PCR for islet hormones and developmental transcription factors in CD44 positive (lane 1), CD44 negative (lane 2), Trk-A positive (lane 3), Trk-A negative (lane 4), and negative control (lane 5) samples. **(B)** Taqman RT-PCR analysis of Trk-A positive (n = 3) and Trk-A negative (n = 3) cell populations showing a significant difference in insulin mRNA expression.

6. Post-sort culture of Trk-A positive and negative cell populations

Viable Trk-A positive and negative cell populations were collected post-sort from two human pancreatic cell preparations and re-cultured as adherent monolayers in collagen coated chamber slides. Immunostaining and quantification immediately post-sort but prior to culture showed the presence of insulin-positive cells only in the Trk-A positive population. Aside from this the phenotypic composition of Trk-A positive and negative populations appeared to be similar with 90-95% of cells expressing CK7 and 60-80% expressing vimentin; another intermediate filament cytoskeletal protein. Cells were seeded at ~0.5 million per well in either RPMI supplemented with 1% ITS and 0.5% HSA alone or the same media further supplemented with 100 ng/ml NGF.

After 24 hours in culture, the majority of cells had adhered to the collagen surface and remained so throughout the culture period (5-6 days). Over this time period, several different culture morphologies were observed. All populations were initially cultured as single cells immediately post-FACS, however upon culture these cells began to re-organize into clusters or cystic structures. Figure 4-5 (Trk-A positive) and Figure 4-6 (Trk-A negative) show the gross cellular organization in these cultures. Many more single cells were present in Trk-A positive cultures (Figure 4-5 A) and those that were present in Trk-A negative cultures appeared more tightly organized with larger cytoplasmic areas (Figure 4-6 A). Groups or clusters of cells were organized as intact, 3-dimensional structures (Figure 4-5 C, 4-6 C) or as tightly packed monolayers with cells at the periphery appearing to be spreading out into the rest of the plate (Figure 4-5 B, 4-6 B). These three different organizations were present in both positive and negative populations but the clustering of cells was much more pronounced in Trk-A negative cultures. Exposure to NGF did not alter the morphological appearance of Trk-A sorted cultures as compared to negative control cultures not exposed to the growth factor.

Immunostaining was performed on Trk-A sorted populations after 5-6 days of culture to determine phenotypic composition. Figure 4-5 shows the presence of insulin positive cells in Trk-A positive cultures either as single cells (Figure 4-5 A) or in 3-dimensional cellular structures (Figure 5C), but not in the cystic monolayer structures (Figure 4-5 B). No insulin positive cells were observed in the Trk-A negative cultures (Figure 4-6 A, B, C) regardless of culture morphology. Trk-A positive cultures at this timepoint were confirmed to still express the receptor by staining with another Trk-A antibody distinct from that used for the original sort (see methods section) (Figure 4-5D). Similarily, Trk-A negative cultures were confirmed to remain absent of the receptor after culture. Finally, continued expression of both CK7 and vimentin was observed after culture, with the majority of cells in both positive and negative cultures expressing these proteins (Figure 4-5 E, F and Figure 4-6 E, F).

Before fixation and immunostaining, cultures were subjected to a static incubation in low and high glucose concentrations to assess glucose stimulated insulin secretion from β-cells present. As expected, no stimulation index (ratio of release in low and high glucose = 1) for insulin secretion was observed in Trk-A negative cultures as no β-cells were present. Surprisingly, Trk-A positive populations also did not respond to glucose stimulation after 2 or 24 hours despite the fact that insulin positive cells were detectable using subsequent immunostaining. Exposure to NGF did not cause a significant change in stimulation index.

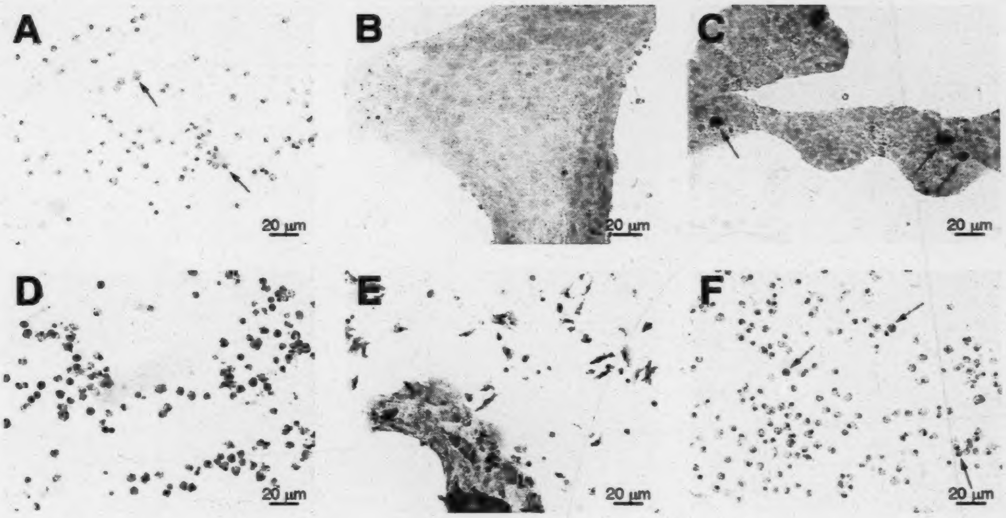

Figure 4-5: Immunostaining of day 5-6 monolayer cultures derived from Trk-A positive sorted cell populations. Hematoxylin counterstaining shows the different culture morphologies observed **(A-C)**. Cultures were stained using the ABC-DAB method (brown color indicates positive staining) for insulin **(A-C)**, Trk-A **(D)**, CK7 **(E)**, and vimentin **(F)**.

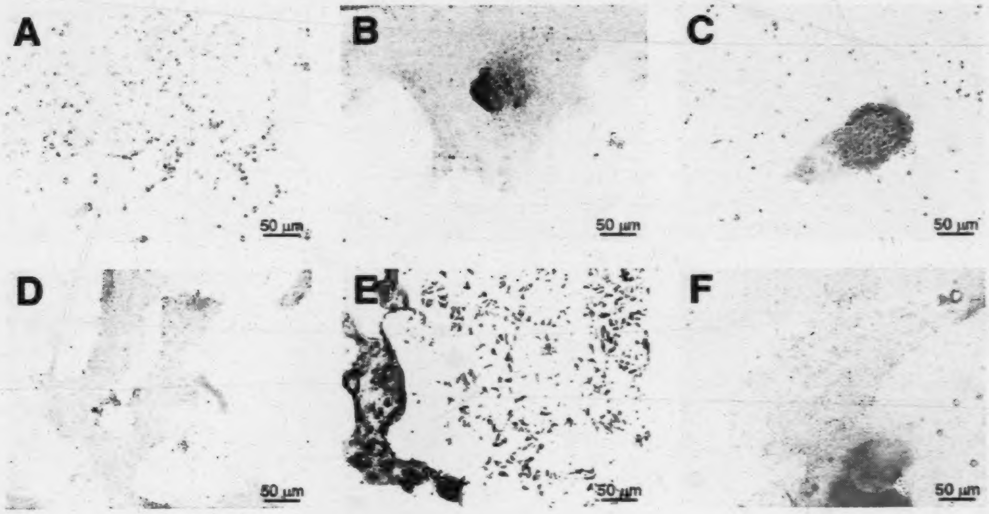

Figure 4-6: Immunostaining of day 5-6 monolayer cultures derived from Trk-A negative sorted cell populations. Hematoxylin counterstaining shows the different culture morphologies observed **(A-C)**. Cultures were stained using the ABC-DAB method (brown color indicates positive staining) for insulin **(A-C)**, Trk-A **(D)**, CK7 **(E)**, and vimentin **(F)**.

IV-D) DISCUSSION

Although pancreatic ductal cells have been widely implicated as islet endocrine precursors (2, 3, 5, 6), it remains to be seen if all ductal cells are capable of islet neogenesis or whether only sub-populations of phenotypically distinct ductal cells have this ability. To address this question, these candidate sub-populations must first be identified and isolated in order to be properly assessed for differentiation potential. This study reports the isolation of two such populations, based on expression of the cell surface receptors CD44 and Trk-A. Furthermore, a preliminary assessment using RT-PCR and immunostaining was performed after isolation of these populations to show differences in gene expression that may be related to islet neogenesis potential.

Clues in the search for an adult pancreatic cell with the ability to form new islets may come from studies of other highly plastic cell populations such as tumours or neoplasms. CD44, the receptor for hyaluronan, is expressed as several possible isoforms and is normally present on epithelial cells and cells undergoing differentiation (22, 31). It has also been shown that CD44 is upregulated during conditions of abnormal growth, such as duct cell (23) and digestive tract (21) carcinomas as well as pancreatic intraductal neoplasms (23). Moreover, another study suggests that hyperplastic neuroendocrine cells associated with pancreatic ducts express CD44 as a sign of de-differentiation (22). For these reasons, we chose to investigate CD44 expression both in the normal adult pancreas as well as in pancreatic ductal cell preparations to examine changes related to plasticity in culture. Interestingly, while intact pancreatic sections exhibited only small focal areas of CD44 expression, immunostaining and FACS analysis of enriched ductal preparations obtained from these organs confirmed an increase in CD44 expression in the range of 30-

40%. While this increase may be due in part to the death of non-CD44 expressing cells and subsequent enrichment of the CD44 expressing fraction in culture, the fact that <1% of the cells before culture expressed the protein suggests that cells not normally expressing CD44 can be induced to express the receptor under abnormal conditions (ie. tissue culture). These results would seem to agree with the upregulation of CD44 in ductal hyperplasias/tumours seen in other studies (21-23), and suggests plasticity in adult pancreatic ductal cells.

The importance of CD44 as a potential islet precursor marker is dependent on whether it is expressed on pancreatic cells of endodermal origin or on mesenchymal cells such as vasculature and connective tissue within the pancreas. A recent study by Hu *et al.* (32) reported the isolation of mesenchymal stem cells from human fetal pancreas based partly on expression of CD44. This suggests that CD44 positive cells in the pancreas are of mesenchymal origin, and may not be islet progenitors. Our results, however, show that CD44 is also expressed on pancreatic ductal cells of endodermal origin, and furthermore that CD44 positive cell populations express the genes for important islet developmental transcription factors such as PDX-1. The presence of mRNA for the islet endocrine hormones and exocrine enzyme amylase in CD44 positive populations shows that some pancreatic endocrine and exocrine cells also express this receptor. In our assessment, a cellular marker present only on either CD44 positive or negative cells was not identified and the significance of CD44 expression on 30-40% of cultured ductal cells remains unknown. It also remains to be assessed whether different isoforms of CD44 are expressed on different pancreatic cell types and what significance this may have.

Trk-A, the receptor for NGF, has been shown to be expressed in the rat pancreas during development (18), as well as on the pancreatic AR42J cell line (16). In addition, islet β-cells have been reported to express both Trk-A and NGF (33, 34), and expression of these proteins may be upregulated by peri-islet Schwann cells in response to islet injury (35). The evidence for a role in islet regeneration/development led us to investigate the expression of Trk-A in the neonatal, juvenile, and adult pancreas, and to attempt to isolate a population of Trk-A expressing cells from adult human pancreatic ductal cell cultures.

Pancreatic biopsies from neonatal, 9 year old, and 54 year old donors were assessed for Trk-A expression. As reported previously in the rat, Trk-A was observed in the immature endocrine and ductal cells of the young developing pancreas (18). However, in contrast to previous studies showing a complete cessation of Trk-A expression in adult animals (18), we show that Trk-A persists in islets and pancreatic ducts of older donors (juvenile and adult). Trk-A positive ductal cells were observed in all biopsies analyzed and were present both in large interlobular and smaller intralobular ductal structures. This also contrasts results found in the scorpionfish endocrine pancreas (36), which demonstrate Trk-A expression only in non-hormone expressing islet cells. Expression of Trk-A in intact human islets appears to co-localize with that of insulin in β-cells in some cases, and in others is expressed in cells not positive for insulin. These differences in expression can be most likely attributed to species-specific differences, and the significance of Trk-A expression in the adult human pancreas has yet to be found.

Because a significant number of Trk-A positive cells were observed in the pancreatic ducts of adult donor biopsies, we attempted to isolate a Trk-A expressing sub-population using FACS from enriched ductal cell cultures. It was found that ~35% of cells in these ductal preparations expressed the receptor after a period of culture, roughly corresponding to the estimated proportion of Trk-A positive ductal cells in pre-culture biopsies. Furthermore, there were no age related differences in Trk-A expression, as cultures from donors <25 years old had a similar proportion of Trk-A positive cells as those from donors >50 years old. This indicates that, although Trk-A expression levels may change during development, this expression remains relatively stable in the pancreatic ducts over adult life.

Although expression patterns during pancreatic development indicate that Trk-A/NGF expression is related to islet formation (18), it remains to be seen whether this holds for Trk-A expressing cells in the adult. The rat derived AR42J pancreatic cell line has been shown to express Trk-A (16), and it is known that this cell line is capable of β-cell differentiation. Our results assessing gene expression in Trk-A positive and negative ductal populations suggests the potential for islet neogenesis, as positive cultures were found to express the genes for both PDX-1 and neuroD. Furthermore, results from real-time PCR show PDX-1 expression levels in the range of that present in an enriched clinical islet preparation, despite the fact that insulin gene expression was several thousand times lower in the ductal preparations (indicating a relative absence of β-cells). This is interesting because in the adult pancreas, PDX-1 expression has been reported to be restricted only to mature β-cells (37, 38). Our results, however, suggest PDX-1 expression in a ductal population and support previous findings by this lab (25) and others (39, 40) that ductal cells in culture express PDX-1 and may be capable of islet neogenesis. Furthermore, another important finding using real-time analysis was that a significantly higher (5-20 fold) amount of insulin mRNA was found in the Trk-A positive as opposed to the Trk-A negative cell population. Although these ductal preparations contain few β-cells to begin with, this data demonstrates that most of the β-cells present do express Trk-A. This confirms previous reports in animals that β-cells express Trk-A and, moreover, shows that adult human β-cells also express the receptor. The fact that such low levels of insulin gene expression were seen in the Trk-A negative population suggests that there were very few β-cells present that did not express Trk-A. Furthermore, immunostaining after sorting also confirmed the absence of insulin-expressing cells in Trk-A negative populations. Thus, Trk-A may become a useful marker as one possible step in the purification of β-cells from other ductal, exocrine, and islet endocrine cells for experimental purposes.

Two Trk-A sorted preparations were cultured after FACS and exposed to a concentration of NGF in the range previously reported to induce cellular differentiation (16, 41, 42). In our hands, this treatment did not result in observable changes with respect to culture morphology, phenotype, or glucose responsiveness. Differences were noted, however, between Trk-A positive and negative cultures. This suggests that although both positive and negative populations were 90-95% CK7 positive (ductal in origin) after FACS, ductal cells expressing Trk-A are different from those not expressing the receptor with respect to growth characteristics *in vitro*. Although a significant number of insulin expressing cells were observed after culture in Trk-A positive populations, these did not exhibit glucose stimulated insulin secretion as assessed by static incubation. This result is unexpected, but could be due to either the existing β-cells losing their glucose responsiveness over a period of culture or to the possibility that many of these β-cells in culture have arisen through islet neogenesis from ductal precursors and are still too immature to respond to glucose.

In summary, we have characterized the expression of two possible islet progenitor markers, CD44 and Trk-A, in the human pancreas. Both receptors were expressed in the adult human pancreas, and moreover significant cell populations expressing either marker were isolated from enriched human pancreatic ductal cell preparations. Although our preliminary assessment of these positive/negative populations did not definitively indicate one to be an islet progenitor population, gene expression analysis showed the potential for islet precursors to be present in both CD44 and Trk-A positive populations. In addition, it was shown that β-cells in these preparations sort almost exclusively into the Trk-A positive population. It is concluded that CD44 and Trk-A may be useful markers in the future for the identification of a phenotypically distinct islet progenitor cell within the adult pancreatic ductal network.

IV-E) REFERENCES

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CHAPTER V

HETEROGENOUS EXPRESSION OF NESTIN IN HUMAN PANCREATIC TISSUE PRECLUDES ITS USE AS AN ISLET PRECURSOR MARKER*

V-A) INTRODUCTION

Recent advances in islet transplantation (1) for the treatment of type 1 diabetes has led to increased demand for a safe, abundant supply of insulin producing tissue. This is an important goal, as presently it often requires islets from two donors to treat a patient using the "Edmonton Protocol", thus limiting the number of recipients for this procedure (1-3). Research has focused on identifying a pancreatic stem cell or islet precursor cell that could be expanded *in vitro* and subsequently differentiated into functional islets. Several possible mechanisms for islet neogenesis have been proposed. These include differentiation from cells residing in the ductal epithelium (4, 5), transdifferentiation of acinar tissue (6-8), and participation of other cell populations expressing specific markers of primitive or undifferentiated cells; for example pancreatic small cells (9) or cells expressing the protein nestin (10, 11).

Nestin is an intermediate filament protein involved in cytoskeletal formation as well as cellular rearrangement to facilitate processes characteristic of undifferentiated cells, such as migration and mitosis (12, 13). It has also been demonstrated that nestin is a cellular marker for multipotent cells in the adult nervous system that can give rise to several neural lineages (14-17). Nestin positive cells have been identified in murine (18), rat (10, 19) and human (11) islets and evidence suggests that these cells can be induced to differentiate into islet endocrine cells (10, 11). Controversy, however, exists over the significance of nestin-expressing cells in islet neogenesis. Some studies report that nestin is not expressed in pancreatic epithelium, but rather in mesenchymal cells (19-22) and thereby suggest the protein does not play a role in islet development. In contrast, other studies show nestin immunoreactivity in rat pancreatic ductal cells (10) and indicate that these are islet precursors. In addition, most studies to date have been restricted to the assessment of nestin expression in the rodent pancreas with few examining its presence in the human pancreas. Therefore, in this study we examined nestin expression within neonatal and adult human pancreatic biopsies to determine the localization of these cells. Moreover, we also evaluated whether nestin-positive cells were present in clinical human islet grafts and assessed its co-localization with other pancreatic cell phenotypes.

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* *A version of this chapter has been published: Street et al. 2004 Journal of Endocrinology 180(2):213-225.*

V-B) MATERIALS AND METHODS

1. Pancreatic biopsies and islet isolation

Human pancreases were procured with informed research consent from donors by the University of Alberta Clinical Islet Transplant Program. Before collagenase digestion of the pancreas, two biopsies were dissected from the head region of the organ and either immediately snap frozen at -196°C and stored at -80°C for subsequent RNA extraction or fixed in Z-fix (Anatech Ltd. Battle Creek, MI). The pancreas was subsequently processed for islet isolation according to the protocols described by this laboratory (1, 23). Briefly, organs were perfused via the duct with the enzyme solution Liberase (Roche, Indianapolis, IN) and once digested, islets were purified on continuous Ficoll gradients using a refrigerated Cobe 2991 (COBE BCT Inc., Lakewood, CA) and assessed for purity by staining with dithizone. Post-mortem neonatal pancreas sections were obtained from the Department of Laboratory Medicine (Anatomical Pathology), University of Alberta Hospital. All protocols used in this study were approved by the Research Ethics Board of the University of Alberta.

2. RNA preparation and PCR for nestin

For frozen tissue biopsies, samples were freeze ground with a mortar and pestle individually in liquid nitrogen (-196°C) and RNA was extracted on Qiagen columns according to the manufacturers protocol (Qiagen Inc., Ontario, Canada). Frozen samples of isolated islet preparations were resuspended in 1 ml trizol reagent (Gibco/Invitrogen, Burlington, Canada) and RNA extracted according to the manufacturers protocol. cDNA was synthesized from 1 μg mRNA with 10 units (200 U/μl) Superscript reverse transcriptase in 1X buffer containing 0.01 M DTT, 0.5 mM dNTPs, and 0.02 μg/μl oligo dT15. For each sample, 2 μl of cDNA was used per 25 μl reaction along with 1X PCR buffer, 2 mM MgCl, 0.2 mM dNTPs, 1 unit taq polymerase (5 U/μl), and 0.5 μM of each primer pair (all chemicals/reagents purchased from Gibco/Invitrogen). For PCR reactions, 35 cycles were performed, with 30 sec. denaturation at 94°C, 30 sec. annealing at 58°C, and 30 sec. extension at 72°C. Final polymerization was at 72°C for 10 minutes followed by a 4°C hold. Products were separated on a 2% agarose gel and images captured on Alpha Digidoc software (Perkin-Elmer, Boston MA). For RT-PCR on all samples tested, two different primer sets were used to confirm positive results for nestin mRNA. Primer sequences for a 496 b.p. fragment of the human nestin gene were obtained from Zulewski *et al.* (10) and were as follows: 5'-AGA GGG GAA TTC CTG GAG-3' (sense) and 5'-CTG AGG ACC AGG ACT CTC TA-3' (antisense). In addition, utilizing a different sense strand (5'–ATG AAC GGG CTG GAG CAG TCT GAG GAA AGT–3') with the same antisense strand from Zulewski *et al.* (10), a 195 b.p. fragment of the human nestin gene was produced from the same samples. RT-PCR for human β-2 microglobulin (5'-CCA GCA GAG AAT GGA AAG TC-3' sense and 5'-GAT GCT GCT TAC ATG TCT CG-3' antisense) was used to verify RNA/cDNA integrity as well as to show the absence of genomic DNA contamination, as only a 268 b.p. fragment was seen upon gel visualization and not the 900 b.p. genomic version of the gene. All PCR products were sequenced and confirmed using a TOPO TA cloning kit (Invitrogen) and blast search against known GenBank sequences (Accession numbers: X65964 for human nestin, and NM_004048 for human β-2 microglobulin). The ED283 cell line (gift from Dr. Anita Gainer, University of Alberta, Edm.) was used as a positive control for nestin mRNA. Negative controls consisted of human cDNA known to be absent of the nestin gene (Applied Biosystems, Foster City, CA) and H_2O in place of experimental cDNA.

3. Immunohistochemical analysis

Pancreatic biopsies fixed in Z-fix were processed, embedded in paraffin, and 3 μm sections were cut and placed on to histobond slides (Marienfeld, Germany). Intact islet preparations were dissociated into single cells by mechanical disruption with a pasteur pipette at 37°C in Ca^{2+}-free media supplemented with 1 mM EGTA and 0.5% BSA for 7 minutes before addition of trypsin (25 μg/ml) and DNAse (4 μg/ml) and further pipetting for 4 minutes. Cells were then allowed to adhere to histobond slides and fixed in Bouin's fixative for 12 minutes before storage at 4°C in 70% ethanol. Immunostaining of single cell and paraffin-embedded sections was performed using both the ABC-DAB and indirect immunofluorescence methods. Sections or cells were quenched with a 20% H_2O_2/methanol solution. Microwave antigen retrieval was performed for 15 minutes on high power (Sanyo household model, 1260W) in 800 ml of 10mM sodium citrate solution (pH 6.0) for paraffin sections or 6 times 5 seconds in 50 ml of 10mM sodium citrate for cell samples. Blocking was performed with 20% normal goat serum (Fisher) for 15 minutes. Primary antibody concentrations were as follows: 1:200 polyclonal rabbit anti-human nestin 331-B (gift from Dr. Conrad Messam, NINDS, NIH), 1:1000 guinea pig anti-porcine insulin (Dako, Denmark), 1:200 rabbit anti-human amylase (Sigma), 1:50 mouse anti-human smooth muscle alpha-actin (Dako), 1:100 mouse anti-vimentin (Dako), and 1:50 mouse anti-human CK19 (Dako). Primary antibody incubations were 30 minutes at room temperature followed by washing (3X) with PBS before addition of secondary antibody. For amylase staining in cell samples, biotinylated secondary antibody was goat anti-rabbit obtained from Vector Laboratories (Burlingame, CA) and used at a concentration of 1:200 for 20 minutes at room temperature. ABC complex (Vector) incubation time was 40 minutes at room temperature and visualization was with the chromagen diaminobenzadine (Biogenex, San Ramon, CA) for 5 minutes. For indirect immunofluorescence, secondary antibodies were 1:200 Cy3-conjugated donkey anti-mouse, 1:200 FITC-conjugated goat anti-guinea pig, and 1:200 Cy3-conjugated goat anti-rabbit (Jackson Immunoresearch Laboratories Inc., West Grove, PA). Double staining was performed in sequential order for both double immunofluorescence and ABC-DAB/fluorescence. For double staining using nestin and amylase antibodies in dissociated cell samples, stripping with 0.01M HCl was performed between addition of primary antibodies to remove excess/unbound antibody and prevent non-specific staining (University of Alberta Histology Dept., 24). Negative controls for nestin immunostaining were performed using rabbit pre-immune serum also provided by Dr. Conrad Messam (NINDS, NIH). Negative controls for all other antibodies were performed by deletion of the primary antibody during the staining protocol. Slides were coverslipped with either Vectashield (Vector) or entellan (EM Science, Gibbstown, NJ). Images were captured on a Zeiss Axioskop II fluorescent microscope with a Coolsnap camera and IP lab software (Scanalytics inc., Fairfax, VA). Single cell counts were performed on a minimum of 500 cells per sample and percentages calculated as number of positive per 500.

4. Statistical Analysis

Values are expressed as mean ± standard deviation. Statistical significance of differences was calculated by a one way analysis of variance (ANOVA) and Scheffes test. Linear analysis of correlations was calculated using Pearsons correlation coefficient and all tests were performed on Statview (SAS Institute Inc, Cary, NC). All significance levels were set at $p<0.05$.

V-C) RESULTS

1. Nestin mRNA expression in human pancreatic biopsies and isolated islets

We initially used RT-PCR to analyze RNA isolated from 5 different adult pancreatic biopsies collected prior to islet isolation in order to confirm expression of nestin mRNA. In Figure 5-1 (lanes 1-5) a 496 b.p. fragment was detected in 5 of the 5 biopsies tested using primers previously described for the human nestin gene (10). A different primer set giving a 195 b.p. fragment was also used to confirm this result (data not shown, see Methods) Subsequent isolation of both fragments and sequencing confirmed this product to be human nestin. Nestin mRNA was also detected in two isolated human islet preparations (Figure 5-1, lanes 6, 7), thereby indicating that cells having the potential to express nestin are retained during the islet isolation procedure. A human glioblastoma cell line was used as a positive control for nestin RT-PCR (lane 8) and negative control reactions using human control cDNA (lane 9) and H_2O (lane 10) showed the absence of the nestin gene product.

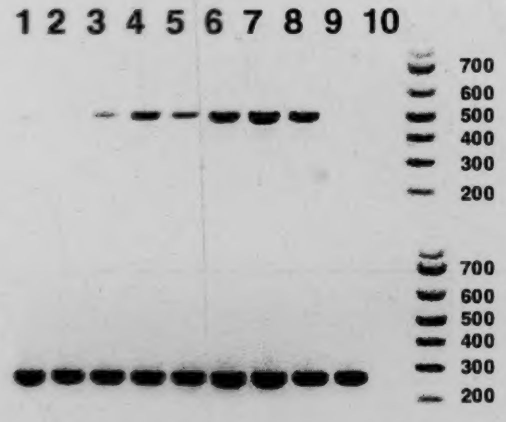

Figure 5-1: (Top) RT-PCR for a 496 b.p. fragment of human nestin mRNA in five pancreatic biopsies prior to islet isolation (lanes 1-5) and in two different isolated islet preparations (lanes 6-7). The ED 283C glioblastoma cell line was used as a positive control for human nestin (lane 8) and negative controls consisted of human control cDNA (lane 9) and H_2O in place of experimental cDNA (lane 10). (Bottom) RT-PCR on the same samples as above for human β-2 microglobulin to verify RNA/cDNA integrity.

2. Nestin localization in human acinar pancreas

Immunohistochemical staining of human pancreatic sections (n = 15) revealed a diffuse and variable pattern of nestin expression amongst different donors. Some pancreases exhibited high levels of nestin expression while others were shown to be significantly lower (Figure 5-2). Differences in the abundance of nestin-positive cells between donor organs were due largely to the relative presence or absence of nestin immunoreactivity in acinar tissue. Extensive nestin staining was present in the acinar lobules of some organs (Figure 5-2 A1, 2) while virtually absent in others (Figure 5-2 B1, 2). Comparisons of all biopsies analyzed revealed a range of nestin expression in acinar tissue from very little (<1%) to extensive (~30%) with 11 biopsies expressing the protein in 10% or more of the cells in a given section and 4 biopsies with less than 10% of the section. In order to more accurately assess the co-localization of nestin with acinar cells we examined dissociated single cell suspensions prepared from clinical islet preparations. These samples were found to contain 34.7±16.1% amylase positive cells, while 40 – 50% of the cells expressing nestin also stained positive for amylase (Figure 5-3 A, B). Arrows on Figure 5-3 A1, 2 demonstrate cells expressing both nestin and amylase (A), amylase but not nestin (B), and nestin but not amylase (C).

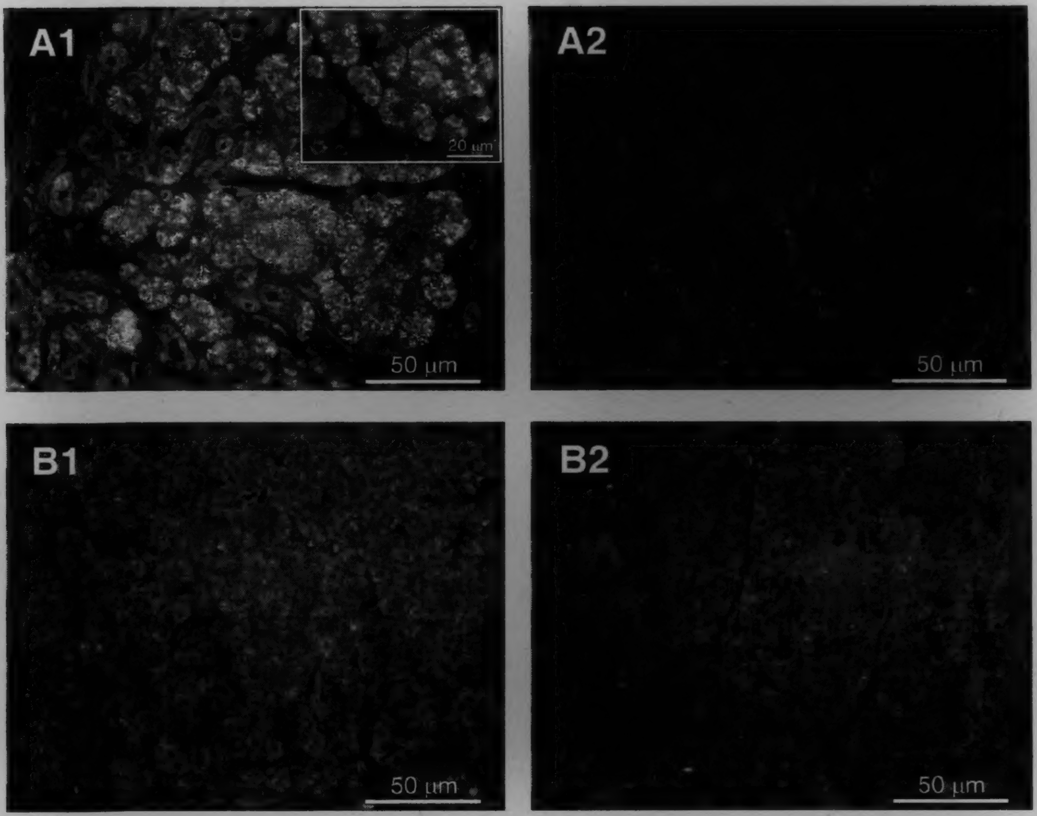
A1
20 µm
50 µm
A2
50 µm
B1
50 µm
B2
50 µm

Figure 5-2: Localization of nestin-positive cells in the acinar component of human pancreatic sections. Nestin was found to be highly expressed in some organs (**A1**; negative control for nestin **A2**) while much lower in others (**B1**; negative control for nestin **B2**).

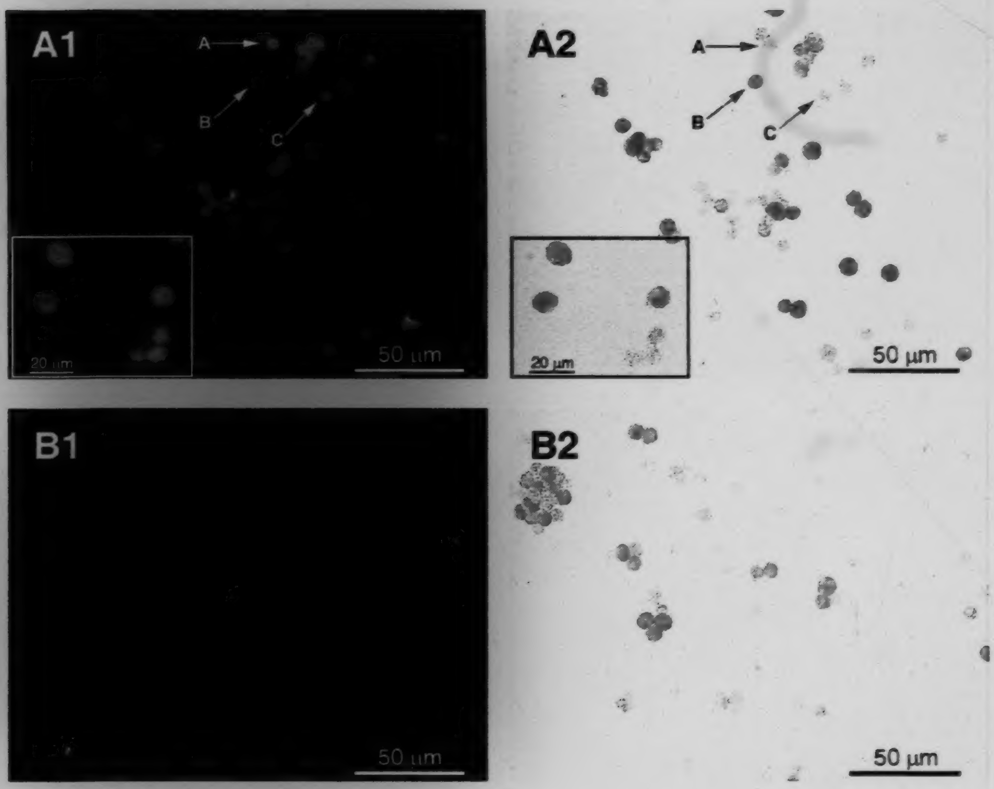

Figure 5-3: Co-localization of nestin and amylase-expressing exocrine cells in dissociated clinical human islet grafts. **(A1, 2)** Same field of cells showing nestin staining (**A1**, Cy3) and amylase staining (**A2**, brown color). Arrows indicate cells expressing both nestin and amylase (**A**), amylase alone (**B**), or nestin alone (**C**). **(B1, 2)** Same field of cells showing the negative control for nestin using pre-immune serum (**B1**) and amylase staining (**B2**).

3. Nestin expression in pancreatic ducts

Nestin-positive cells were observed to be infrequently associated with both small (Figure 5-4 B1, 2)) and larger (Figure 5-4 A1, 2) pancreatic ducts, although these cells typically did not express CK19 (inset, figure 5-4 A1) whereas the majority of cells within the ducts did. However, in some sections, a small proportion of cells were found to express both nestin and CK19 (arrow, Figure 5-4 B1). Analysis of dissociated clinical islet preparations revealed similar results whereby only a small percentage of cells co-expressed both nestin and CK19 (<1%) (inset, Figure 5-4 C1) while a greater proportion expressed either CK19 alone (23.8±11.9%) or only nestin (16.6±13.7%). Figure 5-4 C1, 2 shows staining for CK19 and nestin by dual immunofluorescence.

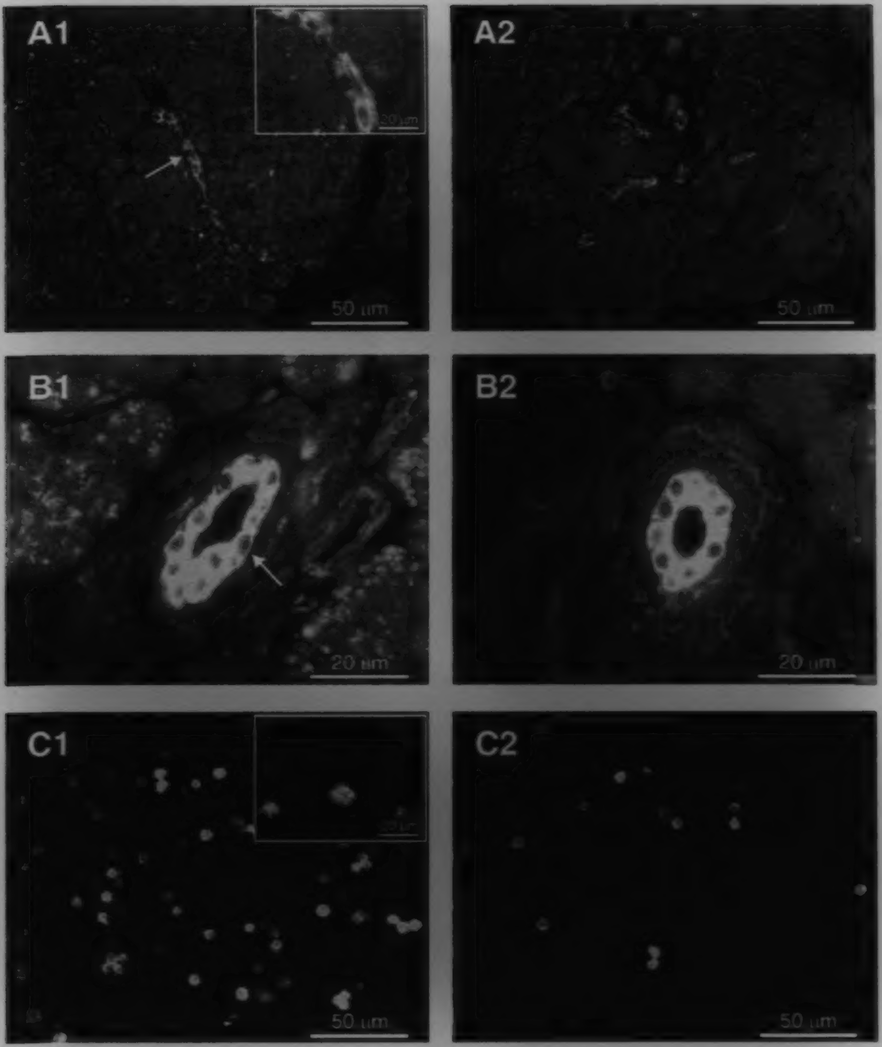

Figure 5-4: Co-localization of nestin (Cy3) and CK19 (FITC) in human pancreatic ducts and dissociated ductal cells. **(A1, 2)** Staining for nestin and CK19 in larger ductal structures. Inset indicates a cell within the duct expressing nestin but not CK19. **(B1, 2)** Staining for nestin and CK19 in small duct structures. Arrow indicates a CK19-positive ductal cell co-expressing nestin. **(C1, 2)** Double staining for CK19 and nestin in a dissociated human pancreatic cell sample. Inset indicates a cell expressing both markers. **A2**, **B2**, and **C2** depict corresponding negative controls for nestin immunostaining using pre-immune serum and regular staining for CK19 to show ductal structures.

4. Nestin expression in islets

Nestin-positive cells were also detected within the majority of islets examined in intact adult pancreatic sections (Figure 5-5). These cells comprised a small percentage of the islet mass (Figure 5-5 A1, 2), and co-localization of nestin and insulin was rarely observed, indicating that nestin is not normally expressed in the mature ß-cell. However, in one pancreatic biopsy, nestin was shown to be co-expressed with insulin (arrow, Figure 5-5 B1, 2) and these rare double-positive cells were usually observed in small clusters of ß-cells or as single ß-cells. Despite this exception, close examination of dissociated clinical islet grafts showed that in almost all cases the nestin-expressing cells (16.6±13.7%) did not co-localize with insulin in mature ß-cells (23.4±11.9%) (Figure 5-5 C1, 2).

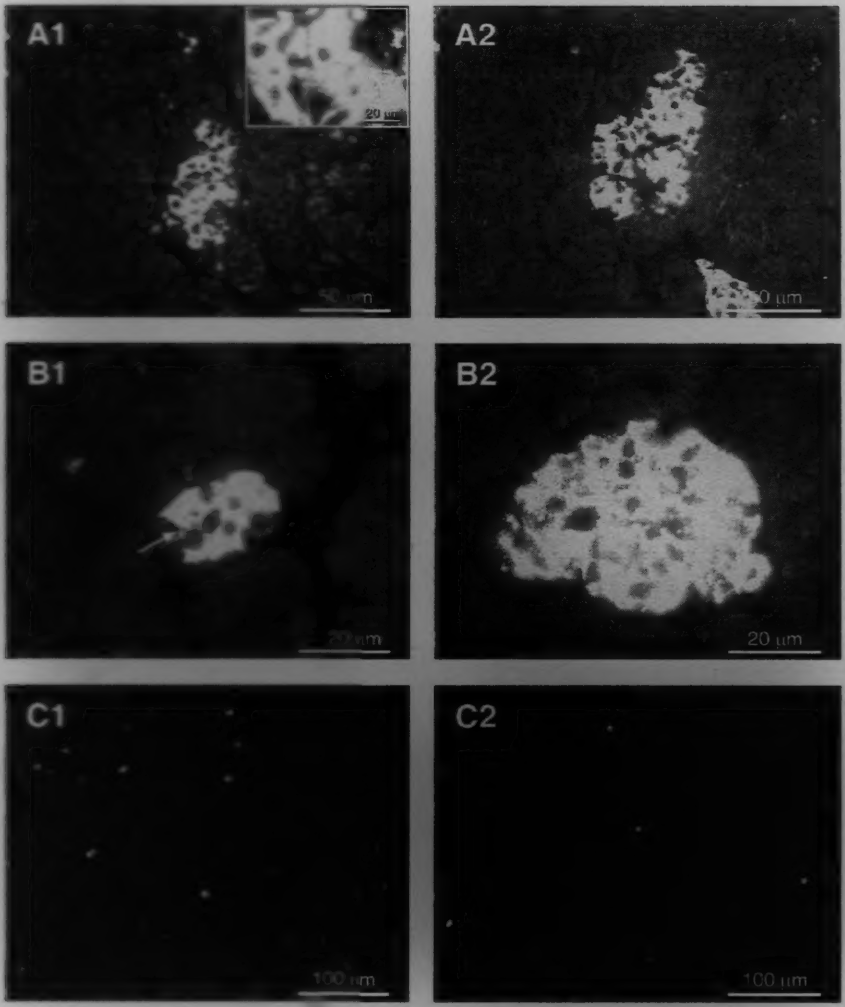

Figure 5-5: Nestin expression in human pancreatic islet sections and dissociated islet preparations. **(A1, 2)** Staining for insulin (FITC) and nestin (Cy3) in an adult pancreatic section indicates frequent intra-islet expression but no co-localization of nestin in β-cells. **(B1, 2)** In rare circumstances, however, insulin and nestin were co-expressed in cells of some small islets (arrow). **(C1, 2)** Double staining in dissociated islet cell preparations for insulin (FITC) and nestin (Cy3) revealed no colocalization of nestin in insulin-positive cells in these grafts. **A2**, **B2**, and **C2** depict corresponding negative controls for nestin immunostaining using pre-immune serum and regular staining for insulin to show pancreatic islets.

5. Nestin expression in pancreatic vasculature and mesenchymal cells

Because several reports have described nestin expression in the pancreatic vasculature and in cells of mesenchymal origin, we further examined nestin expression patterns within the pancreas. Smooth muscle actin was used to identify blood vessels in human pancreatic sections, and our previous experiments demonstrated that CK19-positive pancreatic ductal epithelium does not stain positive for smooth muscle actin (data not shown). Figure 5-6 A1, 2 illustrates characteristic smooth muscle actin staining in large blood vessels in the adult pancreas. Both large and small blood vessels expressed smooth muscle actin ubiquitously, and cells of these structures were often but not always shown to co-express nestin (inset, Figure 5-6 A1). No cells expressing smooth muscle actin were observed in dissociated human islet preparations and thus co-localization with nestin was not observed in these samples. Using vimentin as a marker for mesenchymal cells, nestin expression was also assessed in the mesenchymal component of the human pancreas (Figure 5-6, B, C, D). Frequent nestin expression was frequently observed in intact cellular structures staining positive for vimentin (insets, Figure 5-6 B1, C1), along with single expression of one or the other marker in the same structures. Furthermore, immunostaining of dissociated pancreatic cell preparations showed frequent co-expression of nestin and vimentin (Figure 5-6, D1, 2 and inset). In the human islet preparations assessed, about 20% of the cells staining positive for nestin also expressed vimentin.

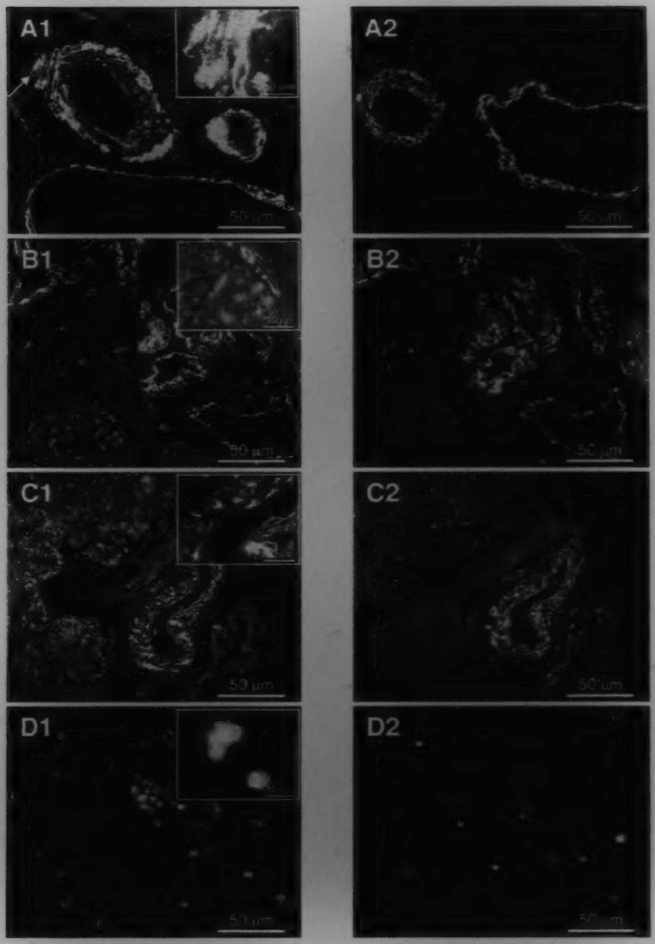

Figure 5-6: Nestin expression associated with pancreatic vasculature and mesenchyme-derived cells. **(A1, 2)** Nestin (Cy3) and smooth muscle alpha-actin (FITC) staining shows nestin expression in vasculature-associated cells. Inset indicates a double-positive cell. **(B, C 1, 2)** Nestin (Cy3) and vimentin (FITC) staining shows frequent co-expression of both markers in vimentin-expressing mesenchymal structures. Insets indicate double-positive cells. **(D1, 2)** Staining for nestin (Cy3) and vimentin (FITC) in dissociated pancreatic cell preparations also shows frequent co-localization of the two markers. **A2**, **B2**, **C2** and **D2** depict corresponding negative controls for nestin immunostaining using pre-immune serum and regular staining for smooth muscle actin/vimentin to show vasculature and mesenchymal cells.

6. Nestin expression in neonatal human pancreas

In order to predict whether nestin plays a role in pancreatic or islet development, expression was also assessed in whole pancreatic sections derived from a neonatal human pancreas. Staining in these sections revealed a pattern similar to the adult, whereby nestin-expressing cells were found associated with the acinar tissue in and near some ductal structures, blood vessels, mesenchymal cells, and also in most islets. One striking difference was the pattern and abundance of staining in the neonatal islets. The neonatal human islets contained significantly more nestin-positive cells compared to adult islets, often localized around the mantle of the islet as well as interspersed through the center (Figure 5-7). Figure 5-7 A1, 2 shows nestin expression in several neonatal islets and Figure 5-7 B1, 2 depicts a typical pattern of nestin staining in a neonatal islet at high magnification (1000X). No insulin/nestin co-expressing cells were observed in the neonatal islets despite this increase in nestin expression.

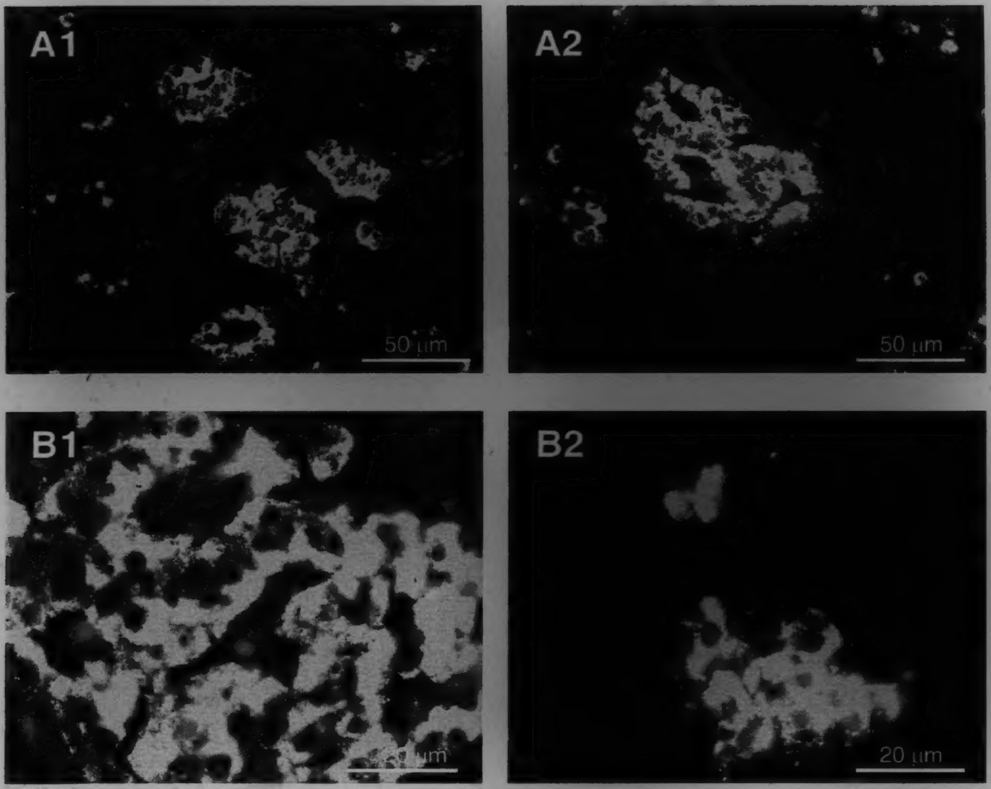

Figure 5-7: Nestin localization in islets of the neonatal human pancreas. **(A1, 2)** Immunostaining for insulin (FITC) and nestin (Cy3) at lower magnification (400X) shows extensive nestin expression in neonatal pancreatic islets. **(B1, 2)** Same staining at high magnification (1000X) shows a high proportion of nestin expressing cells in a neonatal islet interspersed with insulin-producing β-cells. **A2** and **B2** depict corresponding negative controls for nestin immunostaining using pre-immune serum and regular staining for insulin to show pancreatic islets.

7. Nestin-expressing cells in human clinical islet grafts

Immunostaining and quantification of dissociated samples from clinical islet grafts showed a large amount of variation in proportion of nestin-positive cells. Grafts (n = 48) were analyzed retrospectively and characterized as being 23.4±11.9% ß-cells, 9.2±6.2% alpha-cells, 23.8±11.9% ductal, and 34.7±16.1% acinar cells. These grafts were found to have a mean of 16.6±13.7% nestin-positive cells with a range of 0.5% to 72%. This variability between preparations did not correlate with factors such as donor age, gender, body mass, period of cold ischemia prior to islet isolation, or the collagenase digestion time. Similarly, the proportion of nestin positive cells also did not correlate with measures of isolation success such as islet equivalents recovered per gram of pancreas, islet preparation purity, and glucose-stimulated insulin secretion.

V-D) DISCUSSION

The discovery and subsequent isolation of a pancreatic stem cell or islet precursor cell would represent an important step towards the production of an unlimited islet supply for transplantation to type 1 diabetics. Although islet neogenesis has been described in adult pancreatic tissue (6, 25, 26), a consensus has not been reached on the specific identity or phenotype of the precursor cells involved. Recently, attention has focused on the protein nestin as a possible marker for a population of cells in the pancreas that may have the ability to differentiate into endocrine cells (10, 11, 18, 27). We have characterized the expression of nestin in the adult and neonatal human pancreas, as well as in dissociated human pancreatic islet cell grafts, with special attention to its co-localization with other pancreatic cell types. Furthermore, we have defined differences in expression and localization patterns depending on the donor pancreas whereby some organs express relatively high levels of nestin and others relatively low.

RT-PCR analysis detected nestin mRNA in five of five pancreatic biopsies as well as two post-isolation islet preparations. This finding confirms the potential for nestin expression in cells of the human pancreas, and led us to analyze specific localization of the protein using immunostaining of human pancreatic sections and dissociated pancreatic cell samples.

Analysis of multiple sections from human pancreatic biopsies revealed a variable pattern of nestin expression between donors. In particular, some donor pancreases had very high levels of nestin expression while others were very low. Although the possibility cannot be ruled out that expression varies in different regions of the pancreas, our reproducibility of results in multiple sections from each pancreas, and the fact that the biopsy was consistently removed from the same area of the head of the organ suggests that an inherent difference exists in expression levels between donors. Furthermore, using double staining in dissociated pancreatic preparations, it was observed that 40-50% of the cells expressing nestin were amylase-positive acinar cells. In these preparations, a correlation was noted between the proportion of acinar cells expressing nestin and the levels of nestin expression seen in corresponding intact biopsies. To our knowledge this is the first report of nestin expression in pancreatic acinar cells. Moreover, since nestin-positive cells were observed in islets as well as ductal structures with approximately the same frequency regardless of donor, and expression levels varied widely in the acinar component, this suggests that donor variation depends largely on expression in acinar cells.

Nestin expression in cells of the ductal epithelium may suggest a possible role in islet neogenesis, as it is widely believed that the birth of new endocrine cells in the mature pancreas occurs via differentiation of ductal cells (4, 5, 25, 26). Our findings agree with Habener and coworkers (10) in the rat pancreas whereby scattered nestin-positive cells were found in both large and small ductal structures. In contrast to that study however, where it is reported that no nestin-positive cells co-express the ductal marker CK19, our results show a small percentage of cells (1-2%) in the human pancreas that do express both markers. This discrepancy can most likely be attributed to species-specific differences. Our results therefore suggest at least three phenotypically distinct cell populations within the human pancreatic ducts. Most abundant are the CK19$^+$/nestin$^-$ cells that comprise the majority of cells in the ductal structure (90%). Also present are the CK19$^-$/nestin$^+$ cells that occur at a lower frequency (5-10%) and finally the CK19$^+$/nestin$^+$ cells which are rare (1-2%) and not found in most ductal structures.

Previous studies have reported the presence of nestin positive cells within islets themselves. Hunziker and Stein (18) showed the presence of nestin expressing cells in mouse islets while Habener and coworkers isolated these cells from both rat (10) and human (11) islet cultures. All of these studies fail to show the co-localization of nestin and insulin, suggesting that nestin is not expressed in the mature ß-cell. Our results in whole human pancreas conflict with these reports as, in rare circumstances, nestin was found to be co-expressed in insulin-producing cells. These double positive cells were often observed in small clusters of ß-cells randomly distributed throughout the pancreas. It is possible that these nestin-positive ß-cells are the initiation of newly forming islets, since extra-islet endocrine cells have been previously suggested to be indicators of islet neogenesis in pancreatic regeneration models (28). The fact that no nestin-expressing ß-cells were observed in clinical islet grafts using immunohistochemistry is most likely due to the rarity of these cells or to the destruction of these smaller islet structures during collagenase digestion of the organ.

It has been proposed that nestin-positive cells found in the rat and human pancreas are not of endodermal origin but are in fact vasculature cells or other cells of mesenchymal origin (19, 22). Our analysis of human pancreatic sections supports these findings in part, as nestin was shown to be frequently co-localized in blood vessels of both exocrine and endocrine compartments. Since nestin is known to be involved in cellular remodeling processes (12, 13), and we observed that not all vasculature contained nestin-expressing cells, this implies that remodeling of circulatory pathways may be constantly occurring in some areas of the pancreas in response to changing dynamics between endocrine/exocrine cells.

Examination of neonatal or developing pancreas, as opposed to fully matured adult pancreas, may provide insight into the role of nestin in pancreatic development. For this reason nestin expression was also assessed in neonatal human pancreatic biopsies. Although expression was heterogenous in these sections as it was in the adult, surprisingly the neonatal islets contained far more nestin-positive cells than adult pancreatic islets. Selander and Edlund (20) recently reported that nestin-positive cells in the developing mouse pancreas were not epithelial in nature and did not express any endocrine hormones or key transcription factors such as PDX-1 and thus were mesenchymal in origin. Although we observed nestin-positive cells clearly within the islet structure itself, the vast majority of these did not express insulin or the ductal epithelial marker CK19 indicating they may in fact be mesenchymal cells. The fact that these cells also did not co-express smooth muscle actin, however, suggests they are not part of the islet microvasculature. Interestingly, Bouwens and coworkers (7) observed a tenfold increase in nestin-positive cells within islets of a duct-ligated portion of rat pancreas, where an environment of organ neogenesis is created that may be similar to fetal or neonatal development. The authors also report that these cells were found surrounding ducts and islets and proposed them to be involved in angiogenesis to support newly forming islets and exocrine tissue during regeneration. We also observed increased nestin immunoreactivity surrounding islets in the growing neonatal pancreas, and although these were not mature blood vessel endothelial cells, it cannot be ruled out that these cells may be involved in angiogenesis and are not endodermal in origin.

Quantification of immunostaining in clinical human islet grafts showed a wide range in proportion of nestin-expressing cells (<1% - 72%) with a mean of 16.6±13.7%. This range could not be explained by comparison to donor or isolation factors such as age/organ cold ischemia time. Since these human islet grafts are not pure (23.4±11.9% ß-cells), but also contain contaminating acinar cells (34.7±16.1% amylase-positive), and our observations suggest that nestin is co-expressed in acinar cells, it is likely that a large proportion of nestin-positive cells in these grafts are acinar in origin. The possibility also exists that mesenchymal cells are present in these grafts as well, and may be another small source of nestin positive cells. This was evidenced by the extensive co-localization of nestin and vimentin seen in these grafts. Further staining in clinical islet grafts gave results similar to those found in whole pancreatic sections, whereby no cells were found to co-express nestin and insulin, few cells (<1%) co-expressed nestin and CK19, and a large proportion of cells (40-50%) co-expressed nestin and amylase. Analysis of long-term patient outcome may provide answers to the question of significance of nestin-expressing cells in these grafts.

In conclusion, we have shown the presence of nestin-positive cells in clinical human islet grafts and demonstrated that most of the cells in these grafts originate from the exocrine pancreas, with a small number coming from ducts and pancreatic vasculature. The data presented in this study suggests that the majority of nestin-positive cells in the human pancreas are most likely not islet precursors, but rather acinar or mesenchymal cells. Some of the data we report, however, conflicts with a recent study by Klein *et al.* (22), which suggests that all nestin-positive cells of the human pancreas are vascular in origin. This study reports that no endocrine, acinar, or duct cells express nestin and that previously reported duct-associated cells expressing nestin are small capillary structures and do not represent islet precursors. These differing results may be in part due to the specificities of the antisera used in the two studies. It should be noted however, that while the study by Klein *et al.* (22) looked at biopsies from only two different adult pancreases, we have extensively characterized nestin expression in 15 different adult biopsies, 48 clinical human islet grafts, and one human neonatal pancreatic biopsy. Furthermore, we believe that in order to accurately assess co-localization of nestin and other pancreatic markers, it is necessary to examine single cell preparations, as it is often difficult to distinguish double staining with 100% accuracy in intact sections. However, in agreement with the Klein *et al.* study, we did observe that a significant proportion of vasculature-associated cells in the pancreas also express nestin. These observations suggest that nestin cannot be used in the human pancreas as an endocrine precursor cell marker. However, the fact that nestin was observed rarely in extra-islet ß-cells, combined with the observance of increased nestin expression in islets of the neonatal pancreas, shows that the involvement of nestin in islet neogenesis cannot be ruled out without an exhaustive assessment of all populations expressing the protein. Due to its functional role, expression does not necessarily mark a specific cell state or population, but a cellular process that may or may not indicate an involvement in islet differentiation. Isolation, comparison, and clonal analysis of nestin-positive cells from multiple areas of the pancreas will provide further insight into cellular changes in the adult organ.

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CHAPTER VI

ISLET GRAFT ASSESSMENT IN THE EDMONTON PROTOCOL: IMPLICATIONS FOR PREDICTING LONG-TERM CLINICAL OUTCOME

VI-A) INTRODUCTION

The Edmonton Protocol (1) and follow-up studies (2, 3) have shown that islet transplantation is now a therapeutic alternative for some patients with type 1 diabetes. Despite improvements in the isolation of human islets (1, 4, 5), recoveries between donors continue to vary. Assessment of islet equivalents (I.E.) and proportion of dithizone-positive aggregates has been the standard measure used to estimate yield and purity (6, 7), respectively. However, these techniques are not necessarily quantitative largely due to observer subjectivity and there is therefore a need to utilize more accurate methods to assess human islet grafts. Pipeleers and co-workers have shown that islet graft composition with respect to endocrine as well as non-endocrine cell types can influence long term metabolic function of rat islet grafts (8, 9), and have characterized human islet allografts to select the best preparations with respect to endocrine content and donor-recipient compatibility (10).

Graft function is another important factor when characterizing human clinical preparations. Methods to test human islet function include transplantation in immunodeficient mice (4), assessment of insulin biosynthesis (11), *in vitro* perifusion (12), and static incubation (13, 14). The ability to correct hyperglycemia in an animal model is the most accurate assessment, however this requires days to produce an outcome and necessitates the use of a large number of islets. Of the two *in vitro* techniques, static incubation is the fastest and easiest way to obtain an index of insulin secretory activity.

The assessment of clinical human islet grafts in a comprehensive manner will allow the refinement of isolation protocols and the selection of optimal cell preparations for transplant based on comparisons with patient outcome measures. We report the characterization of 83 human islet grafts transplanted to 35 recipients from the inception of the Edmonton Protocol. Preparations were analyzed for endocrine and non-endocrine cellular composition, total cellular insulin content, total cell number, and β-cell function during static incubation. In addition, graft characteristics were compared to donor age and established post-transplant patient outcome measures (2, 3).

VI-B) MATERIALS AND METHODS

1. Islet isolation and transplantation

Pancreases were removed from cadaveric donors with prior informed written consent and stored in chilled University of Wisconsin solution (range 1-15.5 hrs.) before islet isolation. Alternatively, several organs (n=14) were preserved using a two-layer method (University of Wisconsin Solution/perfluorocarbon) (15) for 2-3 hours pre-isolation. Islet isolation was performed as previously described for human islets (1, 5). Upon gradient purification, islets were collected from the topmost gradient layer and transplanted as one preparation, Alternatively, in the less successful purifications, islets were collected separately both from the topmost layer (designated as "top") as well as from the more dense middle layers (designated as "bottom") and these preparations were transplanted sequentially during the same procedure. Immediately after isolation, duplicate samples were taken for immunohistochemistry and dithizone staining as well as insulin and DNA content. Insulin and DNA content was measured using previously described methods (16), and quantification of islet equivalents and assessment of purity was done using a standard islet diameter of 150 µm as previously described (7). In addition, 100-400 µl of the isolated islet preparation was cultured in CMRL 1066 supplemented with 10% fetal calf serum (Sigma-Aldrich, Oakville, ON) and 25 mM Hepes buffer (Sigma) for 18-24 hours before islet function assessment.

Transplant recipients were selected on the basis of labile diabetes, hypoglycemic unawareness, or progressive complications (1). Islets were transplanted immediately or up to 12 hours after isolation according to the protocol developed by Shapiro *et al.* (1). A minimum of 10, 000 islet equivalents per kg recipient body weight were given over 2-3 procedures to 35 patients. Immunosuppression under the Edmonton Protocol (n = 26) was as previously described (1). 8 patients received a modification of this regimen whereby infliximab was given prior to the other medications and 1 patient received a protocol involving Campath-1H for induction, followed by infliximab and maintenance immunosuppression with sirolimus and ultra low dose tacrolimus.

2. Islet graft characterization

Samples taken at the time of isolation were analyzed for cellular composition using immunohistochemistry. Islets were enzymatically dissociated into single cell preparations as previously described (16), and cell suspensions were then placed on positively charged histobond slides (Marienfeld, Germany) and fixed in a water-based Bouins fixative for 12 minutes before storage at 4 °C in 70% ethanol.

Immunostaining of single cell islet samples was performed using the ABC/DAB method (Avidin-Biotin complex visualized with the chromagen diaminobenzidine) as previously described (16). Primary antibody concentrations were: 1/100 rabbit anti-human amylase (Sigma) to identify exocrine cells, 1/1000 guinea-pig anti-porcine insulin (Dako, Denmark) to identify β-cells, 1/50 mouse anti-human cytokeratin-19 (Dako) to identify ductal cells, 1/1000 rabbit anti-human somatostatin (Dako) to identify delta cells, 1/1000 rabbit anti-human pancreatic polypeptide (Dako) to identify PP cells, and 1/1000 mouse anti-human glucagon (Sigma) to identify alpha cells. For quantification of specific cellular phenotypes, single cell counts were performed on a minimum of 500 cells per sample and percentages calculated as number of positive cells per 500. To calculate the cellular mass per graft, total DNA content was divided by 6.6 pg DNA per human pancreatic cell, and then, using the proportions of each phenotype obtained by immunostaining, the absolute number of each cell type was determined. Total endocrine cell purity was calculated by summing the proportions of all 4 endocrine cell types (beta, alpha, delta, PP) in each preparation.

After 18-24 hours culture, islets were recombined in Hams F10 (Sigma) containing a low concentration of glucose (2.8 mM) and washed three times by gravity sedimentation. Duplicate samples were then taken for cellular DNA and insulin content as previously described (16), and the remaining tissue was subjected to a static incubation in Hams F10 containing 2.8 or 20 mM glucose (16). Results were reported as a percentage of the cellular insulin content released, absolute release per 10 I.E., and absolute release per individual β-cell.

3. Patient monitoring and metabolic testing

Pre- and post-transplant metabolic testing was performed on islet recipients as previously described (2, 3). Intravenous glucose tolerance tests (IVGTTs) and arginine stimulation tests were performed between transplants, at 1 and 3 months post-transplant, and then at intervals of 6 months thereafter. The change in AIRg from 1 month to 1 year post-transplant was obtained by calculating the difference between the values for AIRg at these two timepoints. Area under the curve for insulin was calculated as previously described (3) and was the area under the curve above baseline over 30 minutes post-infusion. Patient groups used for long term clinical analysis were selected by the islet transplant program's Endocrinologist (Dr. Edmond A. Ryan, University of Alberta, Edm., Canada) who was blinded to graft function and composition data. For "success" and "failure" groups, mean follow up time was 19.8 and 19.1 months, respectively. Patients in the "failure group" had resumed exogenous insulin therapy at 16.9±3.5 months post-transplant, while all patients in the "success" group remained insulin independent at the time of analysis.

4. Statistical Analysis

Values are expressed as a mean ± standard deviation. Statistical significance of differences was calculated by a one-way analysis of variance (ANOVA) and post-hoc Scheffe's test or unpaired Student's t-test. Analysis of correlations between graft function/composition and donor/isolation characteristics was by Pearson's correlation coefficient and Fishers r to z test performed on Statview (SAS Institute, Cary, NC). Correlation analysis between graft composition and long-term metabolic outcome was performed using Sigma-Stat (Jandel Scientific, San Rafael, CA). Statistical significance for all tests was set at $P < 0.05$.

VI-C) RESULTS

1. Phenotypic composition of clinical human islet grafts

Table 6-1 illustrates the cellular composition and absolute cell number of 83 clinical human islet grafts. The majority of these preparations (n = 69) were both evaluated and transplanted as whole preparations since all gradient tissue layers were combined during the isolation procedure. Alternatively, during some isolations (n = 14), the top and bottom islet layers were collected from the Cobe processor and subsequently assessed and transplanted sequentially. In these cases, values are reported for both layers individually as well as a weighted average given for both combined, taking into account the total cell mass of each fraction.

Table 6-1 shows that the proportion and mass of β-cells in each graft was variable with 23.4±11.9% insulin-positive cells and a mass of $147.4\times10^6 \pm 160.7\times10^6$ cells. The proportion of insulin-positive cells was slightly, but not significantly, lower than that found in top layer alone preparations (28.6±15.3%), although the smaller overall cell mass of the top layer preparations resulted in a smaller absolute number of β-cells ($94.2\times10^6 \pm 94.7\times10^6$). Separated bottom layer preparations, in contrast, showed a significantly lower proportion (7.1±4.9%; $p<0.0001$) and absolute number (21.7±15.5%; $p<0.05$) of β-cells as compared to both top and whole preparations. When values were combined for top and bottom layers, β-cell proportion and number were not significantly different from whole preparations. Similar results were seen when comparing the remaining three islet endocrine cell types.

Grafts were found to contain a significant and variable amount of both exocrine (amylase-positive) and ductal (cytokeratin 19-positive) cells (Table 6-1). Bottom layer preparations were generally less pure as compared to top layer and whole preparations as evidenced by a significantly higher proportion of ductal (36.7±15.5%, $p<0.05$ vs. whole preparations) and exocrine (44.9±9.4%, $p<0.005$ vs. top layer preparations) cells. These differences, however, were not significant when considering the absolute numbers of ductal and exocrine cells present in the grafts due to differences in overall graft mass.

Immunostaining was also performed to examine for the presence of other cell types in clinical islet grafts, such as immune cells and vascular endothelial cells. It was found that <1% of the cells expressed CD45 ($n = 10$) and smooth muscle cell alpha-actin ($n = 10$). For this reason, analysis of these markers was discontinued in the overall graft analysis protocol.

The total mass of each cell type implanted per patient was calculated by adding the individual values over all grafts transplanted. Patients received an average of $320.0\text{x}10^6 \pm 206.5\text{x}10^6$, $132.8\text{x}10^6 \pm 96.5\text{x}10^6$, $63.7\text{x}10^6 \pm 46.0\text{x}10^6$, and $76.2\text{x}10^6 \pm 76.1\text{x}10^6$ beta, alpha, delta, and PP expressing cells, respectively. In comparison, a higher and more variable number of ductal and exocrine cells were received by these patients (ductal: $454.9\text{x}10^6 \pm 306.7\text{x}10^6$, exocrine: $754.0\text{x}10^6 \pm 609.3\text{x}10^6$).

Table 6-1: Cellular composition of clinically transplanted human islet grafts.

		Phenotype					
	n	β	α	δ	PP	Ductal	Acinar
% Composition							
Whole preps	69	23.4±11.9	9.2±6.2	4.0±1.9	4.2±2.9	23.8±11.9	34.7±16.1
Top preps	14	28.6±15.3	10.4±5.8	3.9±1.6	3.8±2.9	29.9±14.8	23.2±13.9
Bottom preps	14	7.1±4.9*	3.9±3.5$^{?}$	2.1±2.5$^{\#}$	5.4±3.5	36.7±15.5$^{\#}$	44.9±9.4**
Combined top/bottom	14	17.7±9.9	7.2±4.5	3.2±1.7	4.5±2.4	33.5±14.1	33.6±9.7
Cell mass/graft (x10^{6})							
Whole preps	69	147.4±160.7	61.5±69.6	29.9±33.3	32.8±41.4	192.6±217.9	346.3±492.1
Top preps	14	94.2±94.7	36.2±50.7	14.0±14.9	13.8±14.9	107.9±97.4	92.7±92.4
Bottom preps	14	21.7±15.5$^{\#}$	11.8±13.7	5.2±3.0$^{\#}$	17.4±14.8	129.1±103.7	161.0±111.5
Combined top/bottom	14	115.9±104.7	48.0±62.1	20.4±17.2	31.2±23.5	237.0±179.9	253.6±196.6
Cell mass/patient (x10^{6})	35	320.0±206.5	132.8±96.5	63.7±46.0	76.2±76.1	454.9±306.7	754.0±609.3

Values are mean ± S.D. of *n* different human islet preparations and are expressed as a percentage of the total cell composition or as the absolute number of cells present in each graft. Whole preparations identify grafts where all gradient tissue layers were collected, transplanted, and evaluated as a single preparation. Top and bottom layer grafts identify preparations where top and bottom gradient layers from one islet isolation were collected, transplanted and evaluated separately. Percentages of combined preparations are calculated by weighting the contribution (in total cell number) of top and bottom from the same donor. * $P<0.0001$ vs. top and whole preparations, $^{?}$ $P<0.05$ vs. top and whole preparations, $^{\#}$ $P<0.05$ vs. whole preparations, ** $P<0.005$ vs. top preparations.

2. Comparison of dithizone-based purity and endocrine purity as assessed by immunostaining.

Islet purity, as assessed subjectively at the time of isolation based on dithizone staining (4, 6) in 82 clinical preparations, was compared to results using immunostaining to quantitate total endocrine cellular composition (as described in Methods section). Dithizone-based purity assessment suggested these preparations to be composed of 66.9±15.8% islets which was significantly higher than endocrine immunostaining results for whole (41.1±16.3%; $p<0.0001$), top layer (46.7±20.1%; $p<0.0005$), and bottom layer (18.1±11.0%; $p<0.0001$) preparations. In addition, a significant difference was seen between total endocrine purity using immunostaining in bottom layer vs. both whole ($p<0.0001$) and top layer ($p<0.0001$) grafts.

3. Mass and insulin content of human islet grafts

Clinical islet grafts were also assessed for cellular insulin and DNA content, and number of I.E. (Table 6-2). Bottom layers contained a significantly lower amount of cellular insulin ($p<0.0005$) than all other preparations, despite the fact that the total DNA content of top and bottom layers was similar. Thus, there was a significantly lower insulin to DNA ratio in bottom layer as opposed top layer preparations ($p<0.01$). Furthermore, the number of I.E. was significantly lower in bottom layer preparations ($63.6\times10^3 \pm 43.5\times10^3$; $p<0.0001$) vs. whole ($344.2\times10^3 \pm 103.3\times10^3$), and top layer ($383.5\times10^3 \pm 158.6\times10^3$), whereas top/bottom combined ($447.1\times10^3 \pm 169.3\times10^3$) grafts contained a significantly higher number ($p<0.005$) of I.E. than whole preparations. The average total cellular insulin, DNA, and I.E. summed over all grafts received by a patient was 8097.6±3164.4 µg, 12064.1±6438.9 µg ($1827.9 \times 10^6 \pm 975.6 \times 10^6$ cells), and $815.9 \times 10^3 \pm 195.2 \times 10^3$ I.E., respectively.

Table 6-2: Composition of clinical human islet grafts.

	n	Insulin (μg)	DNA (μg)	Insulin/DNA	Total Cells (x10^6)	I.E. (x10^3)
Whole preparations	65	3325.8±1915.6	5426.5±5608.7	1.3±1.3	822.2±849.8	344.2±103.3
Separated preparations						
Top layer	14	2930.7±1740.7	2370.1±1988.2	2.0±1.5	359.1±301.2	383.5±158.6
Bottom layer	14	452.2±230.0*	2298.6±1384.8	0.5±0.5$^{?}$	348.3±209.8	63.6±43.5$^{\#}$
Top/Bottom combined	14	3382.9±1843.1	4668.7±3166.6	1.3±1.0	707.4±479.8	447.1±169.3**
Total per patient	35	8097.6±3164.4	12064.1±6438.9	0.9±0.5	1827.9±975.6	815.9±195.2

Values are mean ± S.D. of *n* different human islet preparations or patients and are expressed as absolute numbers or relative ratios. Whole preparations identify grafts where all gradient tissue layers were collected, transplanted, and evaluated as a single preparation. Top and bottom layer grafts identify preparations where top and bottom gradient layers from one islet isolation were collected, transplanted and evaluated separately. * $P<0.0005$ vs. whole, top layer, and top/bottom combined preparations, $^{?}$ $P<0.01$ vs. top layer preparations, $^{\#}$ $P<0.0001$ vs. whole, top layer, and top/bottom combined preparations, ** $P<0.005$ vs. whole preparations.

4. *In vitro* insulin secretory activity

Insulin secretion from islet preparations was assessed during static incubation (14, 16). The insulin secretory activity was not significantly different when comparing whole, top layer, and bottom layer preparations (Table 6-3). Thus, the calculated stimulation indices were also similar. The only significant difference observed was that of insulin release per 10 I.E. at high glucose in bottom layer grafts ($p<0.005$ vs. whole and top layer), however due to a higher basal release at 2.8 mM glucose, this did not make the stimulation index significantly different.

Table 6-3: Insulin secretory activity of clinical islet preparations as assessed by static incubation.

		Insulin Secretory Activity		
	n	2.8mM glucose	20mM glucose	Stimulation Index
% Cellular insulin content				
Whole preparations	66	4.3±5.1	11.2±7.7	4.0±3.9
Top preparations	14	4.5±3.2	12.2±6.9	3.1±1.2
Bottom preparations	14	3.1±3.1	10.5±6.7	4.1±2.3
Release per 10 I.E. (ng)				
Whole preparations	59	2.5±2.5	5.8±4.2	3.7±4.0
Top preparations	14	2.6±2.1	7.4±5.1	3.1±1.2
Bottom preparations	14	3.2±2.9	10.9±7.1*	4.1±2.3
Release per β-cell (pg)				
Whole preparations	31	1.5±1.3	3.6±2.6	3.2±3.4
Top preparations	12	1.2±0.9	3.2±2.5	3.1±1.3
Bottom preparations	12	0.8±0.3	3.0±1.5	4.1±2.3

Values for insulin release expressed either as a percentage released of total cellular insulin content, ng per I.E., or pg per individual β-cell. Stimulation index is expressed as a ratio of insulin release at 20mM glucose to insulin release at 2.8mM glucose. * $P<0.005$ vs. release per 10 I.E. in whole preparations. Three preparations did not contain a sufficient tissue volume after 18-24 hours culture to accurately measure function, and were thus not included in this assessment.

5. Effect of donor age on islet graft composition/function

Isolations from donors less than 25 years of age yielded fewer islets than those from donors 25-50 yrs. and greater than 50 yrs. (Table 6-4), although this difference did not reach statistical significance. However, fewer islet preparations from young donors (<25) were considered suitable for transplantation (20%) than those from the age groups 25-50 yrs. (38%) and >50 yrs. (39%). No significant differences were found in endocrine, ductal, or exocrine mass or cellular proportion regardless of the age group of the donor (Table 6-4). Similarly, although islet stimulation index was lower in preparations from older donors, and insulin content per β-cell was lower in younger donors, neither of these differences reached statistical significance due to the large degree of variation within these age groups.

Table 6-4: Effect of donor age on human islet graft composition and function.

	Donor Age		
	<25 (n = 10)	25 – 50 (n = 31)	>50 (n = 23)
Islet Equivalents ($x10^3$)	290.0±58.9	339.6±104.9	362.6±99.5
Endocrine cell mass ($x10^6$)	251.3±197.4	267.2±219.5	289.7±368.8
Endocrine cell proportion (%)	40.3±15.8	42.8±15.4	40.3±17.1
Ductal cell mass ($x10^6$)	185.8±188.1	193.8±255.0	184.7±181.6
Ductal cell proportion (%)	21.6±7.6	23.3±13.2	25.2±12.2
Exocrine cell mass ($x10^6$)	452.4±578.9	379.5±555.0	245.4±357.3
Exocrine cell proportion (%)	38.7±18.3	35.3±17.5	31.7±13.2
Stimulation Index	4.4±6.7	4.8±4.1	2.8±1.4
Insulin content/β-cell (pg)	23.9±15.9	34.8±25.3	35.1±25.4

Values are expressed either as a percent, absolute cell number, or ratio for *n* human islet grafts derived from donors less than 25 yrs, between 25 and 50, and greater than 50 yrs of age.

R

6. Effect of graft composition on long-term clinical outcome

We compared several established patient outcome measures, including insulin independence and insulin response to arginine or glucose challenge, to islet graft composition and glucose stimulated insulin secretion in an attempt to define a predictor(s) of clinical islet transplant success.

We assessed whether the total number of β-cells transplanted could provide a prediction of short-term metabolic outcome. Figure 6-1 shows a comparison of β-cell number transplanted and both AIRarg and AUCins for the same patient group as was assessed in a previous report with respect to the number of I.E. transplanted (3). Although β-cell number vs. AIRarg shows a positive correlation approaching statistical significance ($r = 0.609$; $p = 0.08$) (Figure 6-1 A1), neither metabolic measure was strongly associated with absolute β-cell number transplanted.

We also attempted to define a correlation between graft cellular composition and long-term transplant success. Two groups of patients transplanted >19 months earlier were selected based on whether they remained insulin independent ($n = 6$) or required subsequent insulin injections at some point after transplant ($n = 5$). These groups were chosen by an endocrinologist blinded to any graft assessment data, and the average time post-transplant for these groups was not different at 19.8 months (success group) and 19.1 months (failure group). One difference observed between these groups was the absolute number of ductal-epithelial cells (CK19 positive) transplanted. Patients in the "success" group were implanted with a total of $500.5\text{x}10^6 \pm 247.5$ (median $435.3\text{x}10^6$) ductal cells whereas those in the failure group received $277.3\text{x}10^6 \pm 224.3$ (median $232.3\text{x}10^6$) ($p = 0.093$). When assessed as "success" and "failure" groups, no difference was seen between the mean number of β-cells transplanted between the groups (276.0±58.2 in success, 421.2±400.2 in failure; $p = 0.40$).

In addition to this analysis, when examining a larger group of patients individually ($n = 19$), a statistically significant positive correlation was observed between the number of ductal cells transplanted and AIRg at approximately 2 years post-transplant (Figure 6-2 B2; $r = 0.490$ and $p<0.05$). In this same group of patients, no significant correlation was observed between the number of ductal cells transplanted and AIRg at only 3 months post-transplant (Figure 6-2 B1; $r = 0.421$ and $p = 0.118$), nor between either the number of β-cells (Figure 6-2 A1, 2) or exocrine cells (Figure 6-2 C1, 2) transplanted and AIRg at 3 months and 2 years post-transplant. Furthermore, no significant correlation was observed between the total I.E. or total pack cell volume transplanted and AIRg at 2 years post-transplant (data not shown). A significant positive correlation was also seen between the number of ductal cells transplanted and the change in AIRg from 1 month to 1 year post-transplant (Figure 6-3; $r = 0.513$ and $p<0.05$).

Post-transplant metabolic outcome was also compared between patients receiving immunosuppression under the Edmonton Protocol and those receiving a modified infliximab protocol. No significant difference in AIRg at either 3 months or 2 years post-transplant was seen between these patient groups.

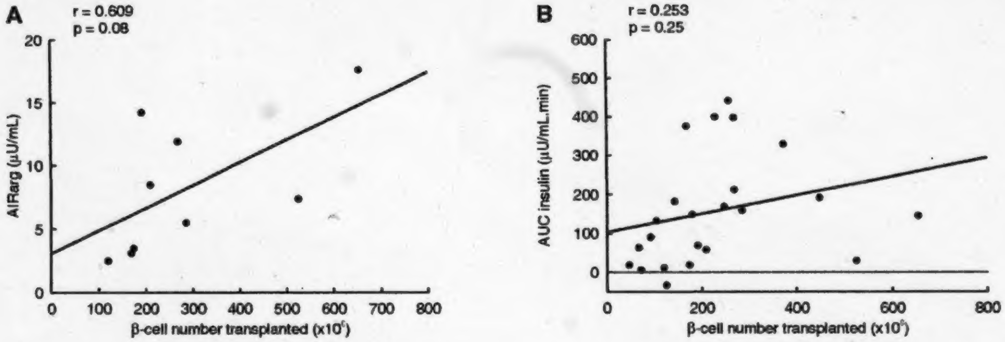

Figure 6-1: Comparison of total β-cell number transplanted and two post-transplant metabolic outcome measures. **A1:** Relationship of the total number of β-cells transplanted and AIRarg as derived from intravenous arginine infusion test at mid-transplant ($n = 6$) and 3 months ($n = 3$) after transplant. **A2:** Relationship of AUCins and the number of β-cells transplanted as derived from IVGTT at mid-transplant ($n = 13$) and 3 months ($n = 13$) after transplant.

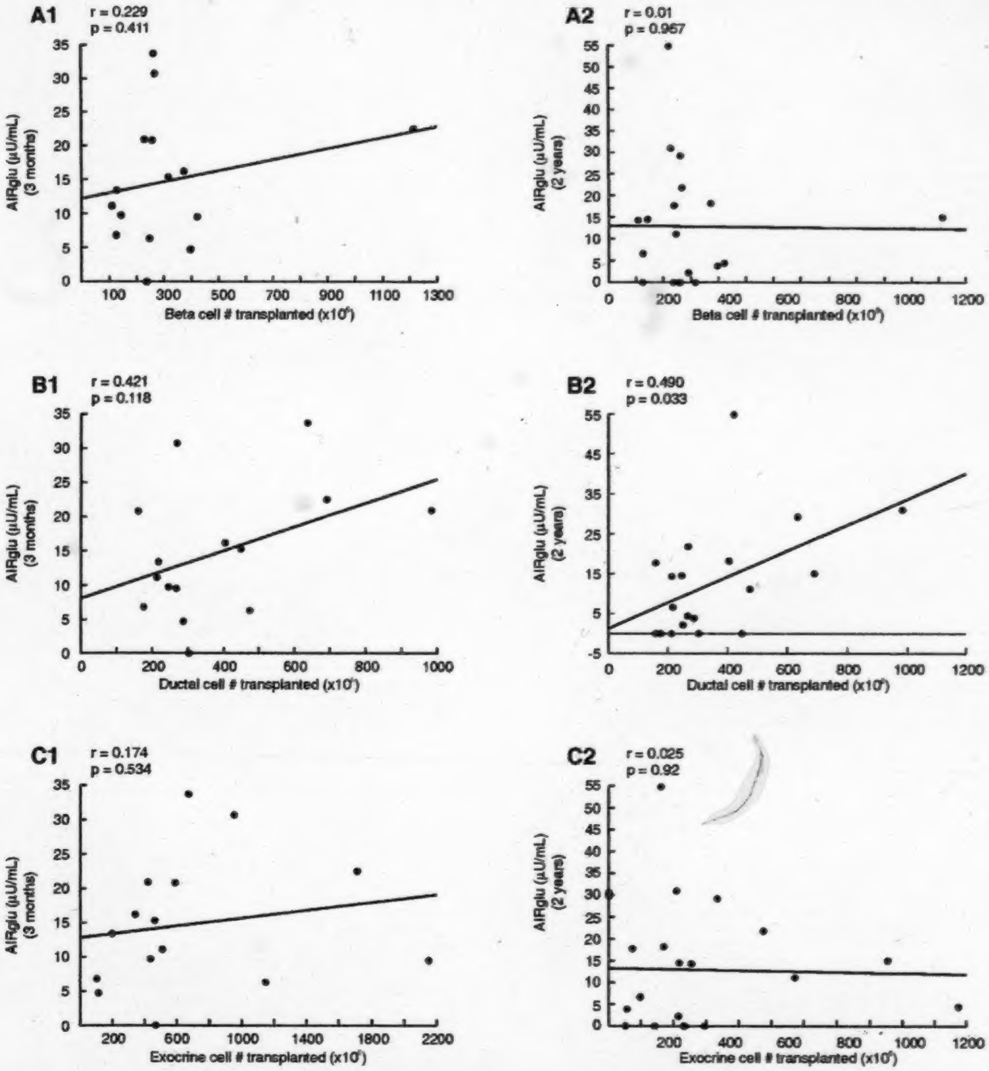

Figure 6-2: Comparison of Graft Composition to Clinical Transplant Outcome. Relationship of β-cell **(A1, A2)**, ductal cell **(B1, B2)**, and exocrine cell **(C1, C2)** number transplanted to AIRg at both 3 months and 2 years post-transplant.

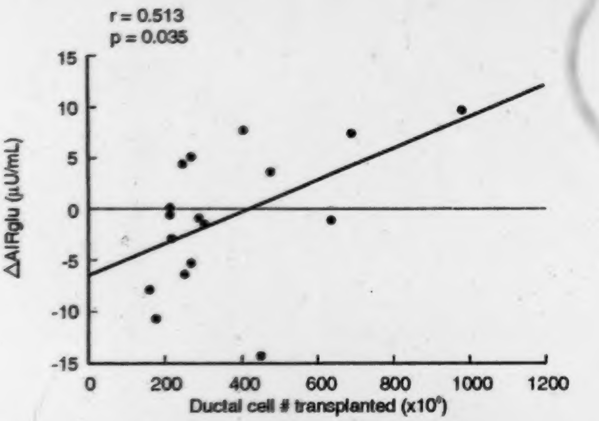

Figure 6-3: Relationship of change in AIRg from 1 month to 1 year post-transplant (n = 17) with total number of ductal cells transplanted in patients receiving islet grafts using the Edmonton Protocol.

VI-D) DISCUSSION

Although methods exist to subjectively assess islet mass (ie. I.E.) and graft purity, few groups are rigorously assessing the cellular composition and function of these grafts. We report data from 83 human islet preparations transplanted to 35 patients beginning with the inception of the Edmonton Protocol. This data was compared donor age and long-term post-transplant metabolic function in recipients to define graft-dependent predictor(s) of subsequent isolation and transplant success.

In our analysis, dissociated cell preparations were used as opposed to staining of whole fixed cellular aggregates (17, 18) in order to more accurately assess the proportion of each cell type. Although, based on dithizone staining, human islet preparations are routinely estimated to be >50% in purity (19), and often approaching 80-90% (4) pure, our results demonstrate this to be an overestimation. In fact, when comparing the overall endocrine purity of the preparations used in this study using immunostaining and comparing to dithizone-based estimations, the difference seen was highly significant. Our results indicate 50% endocrine purity to be an exceptional isolation outcome, even for separated top layer only preparations. Furthermore, our findings indicate an approximate composition of the endocrine component of the grafts of 60% β-cells, 23% alpha cells, 10% delta-cells, and 10% PP-cells. Although PP expressing cells were more abundant in than alpha or delta cells only in bottom layer preparations, overall they were less common and this result is considered to be an anomaly of the specific bottom layer preparations used in this study.

Analysis of cellular composition showed that the majority of islet grafts were composed of non-endocrine cells. Using CK19 as a ductal cell marker (21, 22), it was observed that a significant proportion of ductal cells (20-30%) were present. Furthermore, a large proportion (20-50%) of these preparations consisted of amylase-positive exocrine cells. As expected, the less pure bottom layer preparations contained a significantly higher proportion of both ductal and exocrine cells. Correspondingly, these preparations also had a significantly lower proportion of endocrine cells than the more pure top layer preparations. These findings underscore the need to determine the positive or negative effects of having non-islet tissue present in clinical islet grafts, including the possible presence of islet progenitor cells that could contribute to new β-cell mass through differentiation post-transplant.

Measurement of the total cellular insulin content and total cell number of islet grafts can also give an estimate of purity. Previous studies have suggested the total cellular insulin content of the adult pancreas to be approximately 140 μg/g tissue (23). Assuming an average pancreatic weight of 90 g, this translates to about 12,600 μg total insulin. We obtained an average of 3000-4000 μg insulin in each islet preparation. Therefore, we are either recovering only 25-30% of the β-cell mass, or β-cell de-granulation occurs during the isolation process. Our patients received a total of approximately 8000 μg of insulin, suggesting that, even after undergoing 2-3 transplant procedures, islet recipients receive only approximately 65% of the cellular insulin content present in a normal pancreas. Furthermore, each patient received an average of $320\text{x}10^6$ β-cells over multiple transplants, with an average of 815, 000 I.E., thereby suggesting an average β-cell number of 400 per I.E.

Glucose-stimulated insulin secretion is an important predictor of islet function. Calculating insulin secretory activity as the percentage of the total cellular insulin content released, absolute insulin release per 10 I.E., and absolute release per individual β-cell gave similar stimulation indices in the range of 3-4. This compares with a fivefold average increase reported by Ricordi *et al.* using the perifusion method (4), and is in agreement with an earlier study by Grant *et al.* (24), which also reports a five-fold stimulation index for human islets using a static incubation. Our observations, however, suggest that static incubation data is not necessarily a good predictor of clinical outcome, as no correlations were seen with post-transplant metabolic success. The fact that some successful patients receive islets with poor *in vitro* insulin release (ie. stimulation index<1) suggests that either functional recovery occurs post-transplant, or that the static incubation is not a representative test of islet function *in vivo*. Other tests of function should be evaluated in the future, including perifusion to show a more dynamic biphasic insulin release profile, assessment of insulin biosynthesis, or animal transplantation to test actual *in vivo* function of grafted islets.

It has been proposed that islets derived from younger donors exhibit metabolic advantages for clinical transplantation (23, 25). A younger pancreas contains more insulin per gram of tissue as well as a lower concentration of exocrine digestive enzymes, thus limiting islet destruction by enzymes released from necrotic exocrine tissue during and post-isolation (23). However, isolations from donors aged 30 years or less are technically difficult and yield fewer islets than isolations from older donors (25, 26). We show a similar trend to that previously reported (25, 26), where isolations from donors <25 yrs. yielded fewer I.E., although in contrast to these studies our results did not reach statistical significance. Islet β-cell mass was also examined with respect to donor age and a trend was observed in younger donors, whereby they exhibited a lower insulin content per β-cell, lower number of I.E. recovered per gram of pancreas and a higher stimulation index than preparations from donors aged >50 yrs. It should be noted that a smaller percentage of islet isolations from young donors were considered suitable for transplantation (20% as compared to 38% of 25-50 year old donors and 39% for >50 year old donors). For this reason, the results of this study may be skewed towards indicating less of a difference between age groups, as only the best preparations from younger donor isolations were included in the clinical data set.

Variability in graft cellular composition may be useful as a predictor of clinical transplant outcome. Our group has previously reported (2, 3) that acute insulin response to glucose and arginine indicates both graft mass and function post-transplant. Furthermore, Ryan *et al.* (3) reported a correlation between the number of I.E. transplanted and the metabolic measures AIRg and AUCins. We examined whether the same metabolic measures correlated with the total number of β-cells transplanted in the same patients as the Ryan *et al.* study (3). A correlation was found between AIRg and β-cell number transplanted that approached statistical significance ($r = 0.609$; $p = 0.08$), however there was no correlation between AUCins and β-cell number transplanted. The reason for this discrepancy with the previous study is likely the inaccuracy of the I.E. counting method as compared to immunostaining and quantification of dissociated cell samples.

Several patients under the Edmonton Protocol have remained insulin independent for over 3 years (unpublished data), however in some cases graft failure not associated with immunorejection has necessitated a return to insulin therapy. Finegood *et al.* (27) used a mathematical model to predict an average β-cell life of 1-3 months and proposed a dynamic system of β-cell turnover in the adult pancreas. This suggests that islet cell turnover may also occur in clinical grafts to sustain long-term function after transplant. We examined whether the number of ductal-epithelial cells, which are widely believed to be islet progenitor cells (28, 29), transplanted influences long-term clinical outcome. A difference nearing statistical significance was found between "success" and "failure" groups with regard to the total number of cytokeratin-positive ductal cells received and AIRg at ~2 years post-transplant. Furthermore, a significant positive correlation was observed in a larger group of patients between the number of ductal cells received and both AIRg at 2 years post-transplant, and the change in AIRg from 1 month to 1 year post-transplant. This suggests that a progenitor cell population in human islet grafts may play a role in β-cell turnover and increase long-term graft survival. However, given recent data from Melton *et al.* that suggests that β-cell renewal in the adult may not be from islet progenitors (30), it is possible that ductal cells influence graft survival through another mechanism. For example, ductal cells may secrete factors that exert a protective effect on existing islet cells in the graft, or facilitate improved re-vascularization post-transplant. At this time, however, significant evidence still suggests that ductal cells are islet progenitors (28, 29, 31, 32) and this seems the most likely mechanism for the effect on long-term transplant outcome.

In conclusion, we have provided a summary of human islet grafts with respect to cellular composition, mass, and function. A discrepancy has also been shown between dithizone staining to estimate purity and the more quantitative immunostaining of representative dissociated cell samples. In addition, we have shown that islet graft assessment may provide information about subsequent transplant outcome in that the presence of ductal cells may improve long-term metabolic outcome. It is important to note, however, that significant variability exists in islet isolation success, graft characteristics, and patient outcome. Thus, it remains difficult to accurately define characteristics of donors or graft composition that affect clinical outcome. However, with the improvement and standardization of islet isolation techniques, in the future detailed information collected on human islet grafts will allow the refinement of clinical islet transplantation procedures to build on the success of the Edmonton Protocol.

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CHAPTER VII

GENERAL DISCUSSION AND CONCLUSIONS

VII-A) GENERAL DISCUSSION

The development of the Edmonton Protocol in 2000 (1) has ushered in a new era for islet transplantation. That study and subsequent follow-ups (2, 3) have shown that type 1 diabetes can be controlled using a relatively non-invasive procedure, potentially eliminating the need for exogenous insulin therapy. One of the most promising aspects of islet transplantation is the fact that it offers the potential for a reduction of major long-term complications of type 1 diabetes through a more strict control of daily blood glucose fluctuations. In addition, islet transplantation is a much safer procedure than whole pancreas transplantation; with minimal recovery time needed and relatively minor post-operative complications (4). For these reasons, islet transplantation is now a real therapeutic alternative for certain patients with longstanding, complicated type 1 diabetes.

Despite the much-hyped successes reported for islet transplantation, several major limitations continue to exist that must be addressed before this treatment can be offered as the standard in diabetes therapy. As with all whole organ allograft transplants, there is a need for constant and aggressive immunosuppression in recipients in order to circumvent rejection of transplanted islets. This precludes the use of islet transplantation in young patients, as the risks of this immunosuppression outweigh the risks associated with disease management with daily insulin injections. Newer concerns have also been raised regarding the maximum length of islet graft survival as well as apparent defects of alpha-cell function in transplanted islets (5, 6), raising the risk of severe hypoglycemia should a transplant recipient be required to resume exogenous insulin treatment. Some of these problems may relate to transplant site, absence or delayed vascularization of islet grafts compared to whole organ transplants, or absence of cell turnover from adult progenitor cells post-transplantation. Finally, there is a major problem related to islet availability for transplantation that is only exacerbated by limited organ donation in Western cultures, inefficient techniques for islet isolation, and the growing prevalence of type 1 diabetes in society. For this reason, a major focus of diabetes research is now to develop an alternative source of insulin-producing tissue for clinical transplantation.

Although many sources have been proposed as solutions to the problem of islet supply, in the last few years several possibilities have emerged as the most promising. Modifications to the islet isolation procedure (7, 8) have improved yields and the single donor:single recipient barrier appears ready to fall (9). This, however, cannot on its own cover the needs of all current as well as future persons afflicted with type 1 diabetes. Genetic engineering of the complete islet cell machinery in other cell types or cell lines faces significant technical and theoretical challenges, and therefore at this time is not a feasible alternative. Xenogeneic approaches, specifically the use of porcine islet tissue, remain one of the most promising approaches going forward. Immature neonatal porcine islet tissue is readily available and has been shown to be effective for diabetes treatment in small animal models (10, 11). Further research is now extending the use of neonatal porcine islets into large animal models and this approach will likely move to clinical trials in the foreseeable future. Several barriers, however, still exist for xenotransplantation including the controversy over potential transfer of porcine viruses to the human genome, the increased risk of immunorejection, and general public concern over the use of animal to human organ transplants. Finally, a great deal of attention has focused on the possibility that adult stem cells or adult pancreatic progenitor cells could be used to create new islets. In general, this approach poses fewer ethical and technical barriers than other possibilities mentioned above, however several issues must be addressed before it can become reality. First of all, these putative islet progenitors must be identified and isolated from adult pancreatic tissue for further analysis. Secondly, models must be developed to reliably induce islet differentiation either *in vitro* or *in vivo* from these cells. Thus the focus of this thesis was twofold. We attempted to both identify an islet progenitor cell population from a readily available abundant source of non-endocrine human pancreatic tissue, and to provide evidence that these progenitors can indeed differentiate to β-cell phenotype under the appropriate conditions.

While definitive differentiation of adult pancreatic progenitors to islet phenotype appears straightforward in theory, in practice this has proven exceedingly difficult. A large body of evidence suggests that cells in or associated with the pancreatic ductal epithelium are islet precursors (12-16). Several studies have in fact reported limited islet differentiation *in vitro* from pancreatic ductal tissue. Bonner-Weir *et al.* (13) cultured human ductal cells under defined conditions and reported the presence of glucose-responsive, insulin expressing cells. The problem with this study and others of its kind is that there is always a certain proportion of islet tissue present initially in these preparations that is not taken into account. When these cultures are initiated from gradient purification layers after pancreas digestion that may be rich in islet tissue (as in 13, 17), this may confound subsequent results indicating differentiation. For this reason, our first goal was to develop a model for the derivation of adult human ductal cells from the most "non-endocrine" portion of pancreatic digest (ie. most dense tissue layer). In order to assure that this ductal tissue could also be useful in a clinical model, this culture model was designed to be xenoprotein-free. Building on other models of this type (18-20), we succeeded in obtaining a better yield and higher enrichment than previously reported (18) in a controlled, serum-free environment. In addition, these ductal enriched cultures were characterized with respect to overall cell survival, cell death, insulin content, and gene expression. Importantly, we showed them to be very low in β-cell/islet content (although not completely free) and demonstrated the age-dependent expression of the islet developmental transcription factor PDX-1 in cultured adult human ductal cells.

In Chapter 2, attention was focused on using these enriched human ductal cultures in experiments to induce islet differentiation. A recent study by Heremans *et al.* (21) reported that human ductal cultures adenovirally induced to express the islet developmental transcription factor neurogenin 3 initiated transcription of other downstream factors and underwent a limited amount of differentiation to insulin-producing phenotype. Using a similar approach, we attempted to first reproduce the results from that study *in vitro*, and secondly to design a novel *in vivo* protocol for the differentiation of ngn3 expressing human ductal cells to insulin production. Although many of the results related to gene expression initiation were reproducible in our hands, we could not induce the full differentiation of these cells *in vitro*. In contrast, using a model of intrapancreatic transplantation of ngn3 expressing ductal cells to diabetic immunocompromised animals, we were able to drive full differentiation and showed the presence of an increased number of insulin-expressing cells *in vivo*. Although differentiation was not sufficient to ameliorate the diabetic state in these animals, this study provides good evidence that human ductal cells can be directed to islet differentiation under the appropriate conditions.

After the establishment of "proof of principle" that cells within adult human ductal preparations can undergo differentiation to insulin-producing phenotype, a subsequent aim was to identify which cells are in fact islet progenitors within these populations. Debate has surrounded the question of specific phenotype of an adult pancreatic stem/progenitor cell. Proposed markers for these cells include the proteins nestin (22, 23), ngn3 (24), and islet neogenesis associated protein (INGAP) (25). Based on the literature available to date, we attempted to define an adult human pancreatic progenitor cell within a ductal population based on expression of certain cellular markers/receptors. The ability of selected ductal cells to undergo islet neogenesis may in fact be dependent on expression of receptors for specific growth factors and the cells' ability to respond to stimulation by these factors. Several growth factors have been reported to stimulate pancreatic cells in culture. Epidermal growth factor has been shown to promote proliferation and expansion (26), while the hormones betacellulin (27), keratinocyte growth factor (28), retinoic acid (29), and nerve growth factor (30) have all been reported to induce pancreatic cell differentiation. We chose to analyze expression of the receptors CD44 (ligand: hyaluronan) and Trk-A (ligand: NGF) in human ductal cells to identify potential progenitor subpopulations. CD44 expression has been previously reported in pancreatic tissue and is known to play a role in cell growth and differentiation (31, 32). Similarly, Trk-A has also been implicated in the process of fetal pancreatic development (33, 34) and induction of insulin production in pancreatic cell lines (30). We have identified for the first time both CD44 and Trk-A expressing human ductal subpopulations. It was found that these cells are induced to express CD44 only after a period of culture, although the significance of this expression remains unknown. A substantial proportion of human ductal cells in culture also express Trk-A, and furthermore all residual β-cells present also expressed the receptor. This finding may be significant in that if most or all mature β-cells express Trk-A, the ductal cells that also express Trk-A may represent undifferentiated cells capable of being stimulated to undergo β-cell neogenesis.

Recently, a great deal of controversy has centered on the neural stem cell marker nestin as a potential marker for islet precursors (35-37). In Chapter 5, we assessed nestin expression in the adult human pancreas, as well as in clinical human islet grafts and neonatal human pancreatic sections. It was concluded from these results that nestin, on its own, cannot be used as an identifying marker for islet precursor cells. The main reason for this conclusion is that nestin expression was observed in many different cell types including acinar, ductal, islet, and vascular structures. It must be stressed from these results, however, that expression of nestin does not necessarily exclude a cell from being an islet progenitor, as nestin was shown to be co-expressed in some ductal cells and present at higher levels in neonatal human islets.

Finally, in a separate but related study, we assessed human clinical islet grafts transplanted using the Edmonton Protocol for both composition and function in order to gain insight into predictors of transplant outcome. Previous studies have stressed the need for rigorous assessment of islet grafts to determine islet purity/content (38, 39) and to predict potential immunogenicity (40). In our studies, we were more interested in accurately conveying total graft composition with respect to all cell types present and assessing islet progenitor cell content. In doing this, we showed a discrepancy between the traditional, highly subjective, method of estimating islet purity based on dithizone staining and the more quantitative method of immunostaining dissociated cell samples. It was also observed that *in vitro* glucose-stimulated insulin secretion as assessed by static incubation is not a good predictor of islet graft quality and subsequent transplant outcome. In the future, it may be more meaningful to assess islet function using either perifusion or animal transplantation models to more accurately predict clinical success. With respect to progenitor cell content, we have shown that the number of ductal cells received by a patient over all transplants correlated significantly with long-term graft function as defined by acute insulin response to glucose. This clinical data provides further evidence supporting our animal model differentiation results for *in vivo* islet neogenesis/turnover from transplanted adult human ductal cells.

VII-B) CONCLUSIONS AND FUTURE CONSIDERATIONS

Since the success of the Edmonton Protocol, a great deal of research has been undertaken to try to understand the mechanisms of islet turnover in the adult pancreas. It is expected that this understanding will lead to the exploitation of pancreas-derived progenitor cells to create an abundant source of islets for transplantation. This thesis has contributed to this research focus through the study and manipulation of adult human pancreatic tissue. We have provided evidence, both experimental and clinical, that adult human ductal cells can and do undergo β-cell differentiation under the appropriate conditions. This work is an important stepping stone toward the future goal of using adult pancreatic progenitor cells to create new islets.

Several important considerations exist going forward with these types of studies that should be mentioned in conclusion. First of all, it is imperative that researchers find a way to increase the efficiency of the limited amount of islet differentiation seen in all studies to date. No protocol has yet been developed to produce a clinically relevant supply of insulin-producing tissue that shows normal function (ie. glucose responsiveness). One strategy to potentially improve this is to narrow down a specific ductal-progenitor subpopulation and enriching these cells before inducing differentiation. There should also be a more strict overall focus on analyzing the starting material used in these differentiation experiments. Our studies were undertaken using the most islet-free preparations possible, unlike other recent studies (13, 17). Furthermore, even using our preparations, the need for assessment of residual islet content is apparent before an interpretation of islet neogenesis can be made. Another important issue going forward is the need to characterize pancreatic ductal cells themselves with respect to both function and *in vitro/in vivo* effects on existing islets. New evidence is emerging that ductal cells may secrete cytokines and other substances under certain conditions that may be harmful to islets (41-43). Although our clinical evidence suggests that ductal cells benefit islet graft function in the long-term, it must be investigated whether they may have initial deleterious effects when co-transplanted with islets and what significance this may have for initial engraftment and survival. Again, it is probable that only a subpopulation of ductal cells are in fact islet progenitors and thus the remaining non-progenitor fraction of ductal cells would not be necessary to transplant. This could potentially cut down on the volume of tissue transplanted as well as harmful effects of ductal cell secretions on islets while still providing the possibility of islet turnover post-transplant. Finally, as researchers focus on the induction of β-cell differentiation, the overall goal of creating a fully functional, multicellular islet structure should not be forgotten. It is known that other islet endocrine cell types are important for feedback on β-cells and proper islet function. Although the creation of a pure β-cell population from progenitor cells would be an important experimental advance, it will be necessary (and most likely exceedingly difficult) to re-create the complex islet architecture if this approach is to be successful in the long run.

VII-C) REFERENCES

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